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AN OUTLINE OF THE BALANCE HYPOTHESIS OF PARASITISM[1]

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The present hypothesis is an attempt to explain host-parasite relations in terms of the nutrition of the parasite and the biochemistry of the host. The gist of the idea can be put in a few sentences. Among the host's metabolites available to a potential parasite there are some substances that may favor and some substances that may hinder the growth of the parasite. These substances are vitamins, amino acids, organic acids, and others; they are the substances common to the metabolism of many different species. The kinds and concentrations of metabolites in a host vary as conditions change. This variation, in substances capable of affecting the growth of a parasite, may account for the changes in resistance and susceptibility which sometimes occur within a single host. In different environments the individuals of a host variety may likewise vary in the concentrations of metabolites present and thus the variety may be susceptible in one set of conditions and resistant in another. Among different hosts the kinds and concentrations of metabolites vary from variety to variety and from species to species. These variations plus the different responses of different parasites can be expected to account for the complex patterns of varietal and species resistance and susceptibility. It is conceived that, in general, innate immunity is determined by the normal host metabolites that are present and unfavorable to the growth of the parasite, or by the partial or complete absence from the host metabolites of substances that are required by the parasite, or by a combination of these. Immunity of this sort is not determined by special substances or mechanisms. The metabolites of a susceptible host do not inhibit, but rather favor the growth of the parasite. One can imagine combinations of metabolites that would permit any intermediate condition between complete resistance and complete susceptibility. There is, in a sense, a sort of balance in the host between the substances that inhibit and the substances that promote the growth of a parasite. From host to host or from one host condition to another, the balance may tip toward resistance on

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one hand, toward susceptibility on the other, or remain between the two possible extremes.

In some cases the outcome of a host-parasite relationship can be ascribed to known factors such as antibodies, phagocytes, structural barriers, antibiotics, toxins, and others. For the present, cases of this sort will be considered as exceptions because in terms of these factors only a small portion of the known host-parasite relationships can be understood, and these only partially understood.

The balance hypothesis is an attempt to formulate a common denominator for the examination of the problems of species and varietal resistance and susceptibility, of physiologic races, of elective localization, of virulence, and of changes in resistance or susceptibility due to age, diet, or modified host environment.

Some pertinent, general biochemical considerations which formed the basis for the hypothesis are reviewed in the next two paragraphs and the term "definitive nutrition" is introduced.

According to Snell (1949) foods may be classified as essential, stimulatory, inhibitory, and apparent. Growth occurs only in the presence of essential foods; the number required may be large or small depending upon the organism. Stimulatory foods increase growth but are not essential. A substance that serves as a food for one organism may inhibit another or may, under other conditions, inhibit the organism for which it once served as a food. An apparent food is one that is essential in one environment but not required in another. Even though an organism will grow on a simple synthetic medium, these four types of foods can be found if the complexity of the medium is increased and different combinations of nutrients are tried (Dewey, Parks and Kidder, 1950; Knight, 1936; Snell, 1949; Washburn and Niven, 1948).

Since it is impossible to test all the combinations of kinds and quantities of growth-promoting and growth-inhibiting substances in studying the *in vitro* growth of a parasite, the "definitive nutrition" will be considered as that portion of the parasite's complex nutrition in terms of which it is possible to understand the outcome of the relationship between parasite and host. It will include both the promoting and the inhibiting substances. In general, among a host's metabolites all the types of foods considered above are available to its potential parasites. Because of this, the host supplies a nutritional environment of much greater complexity than is present in the minimal medium of a parasite.

THE NUTRITION HYPOTHESIS

In order to consider well the present concept, it is necessary first to review the nutrition hypothesis of parasitism and to justify the violation of the principle of parsimony.

The nutrition hypothesis states that a parasite grows in a particular host or organ because in that host or organ there is available to the parasite the kinds and quantities of foods that are necessary for its life, and in resistant hosts

or organs the necessary kinds and/or quantities are not present (Bacon, Burrows and Yates, 1951; Francis, 1948; Gäumann, 1950; Leach, 1919). The possible combinations of foods present plus the capacity of the parasite to respond differently to these combinations accounts for the varying degrees of resistance and susceptibility not explicable in other terms.

This is a straightforward and reasonable concept, but there is not yet one case where it has been thoroughly tested by examining the definitive nutrition of a parasite and the relevant content of its host in as complete detail as present biochemical knowledge permits. There are a number of possible reasons for a lack of this kind of research: the overpowering sway of the antigen-antibody concept (Jordan and Burrows, 1946; Chester, 1933); the difficulties of determining the complex nutrition of a parasite; the feeling that assays of the host do not give a true picture of the nutrients that are available to the parasite; the similarity of the *in vitro* nutritional requirements of parasites having different hosts; the failure to correlate pathogenicity with nutritional complexity (Dubos, 1948; Leben and Keitt, 1948); and the impact of the concept of the biochemical unity of life. Most of these are clear, but the last needs a comment. It is the common belief today that all organisms have in their metabolism a great many of the same substances. Thus if all organisms contain substances required by a parasite, what accounts for species and varietal resistance and susceptibility? Obviously it cannot be nutrition. This argument could lead to a rejection of the nutrition hypothesis.

The recent work of Bacon, Burrows and Yates (1951) adds support to the nutrition hypothesis. They found that PAB-less and adenine-less mutants of *Bacterium typhosum* were much less virulent to mice than was the parent strain Ty 22. The virulence of either mutant was equal to Ty 22 if PAB or adenine were injected with the appropriate mutant. The full virulence of the PAB-less mutant was restored if additional PAB was included in the diet of the host.

FACTS AND ARGUMENTS FOR THE BALANCE HYPOTHESIS

Since the nutrition hypothesis has hardly begun to be explored, why does one with any sense of economy propose a more complicated scheme? The most important reason is that wherever one turns in the field of nutrition one finds inhibitions caused by nutrients which are present in metabolic concentrations. Is it not reasonable to assume that, where metabolites of two organisms are intermixed as they must be in the host-parasite relationship, the substances which inhibit growth can affect the parasite equally as well as can the growth-promoting substances? A second reason arises from the results secured when testing the nutrition hypothesis. There has been no correlation between growth requirements and pathogenicity of parasites (Dubos, 1948; Leben and Keitt, 1948). These difficulties have arisen from a neglect of the inhibitory effects of common metabolites which may be present in the complex environment supplied by the host.

There is considerable work in the literature which supplies supporting, circumstantial evidence for the balance hypothesis, but only two papers furnish evidence which may be considered as direct. Since the definitive nutrition of no parasite has yet been worked out, it cannot be expected that the available facts will be decisive. A few of the relevant papers are noted here.

Gladstone (1939) found that valine, leucine, or isoleucine would inhibit growth of *Bacillus anthracis* when added to a basal medium that did not contain any of these amino acids. Leucine and isoleucine together also inhibited growth, but valine and isoleucine caused more rapid growth than occurred on basal medium. When all three amino acids were added to the basal medium, growth was more rapid and more abundant than on basal medium.

Weissman and Graf (1947) have shown that calf thymus histone will stop oxygen uptake in suspensions of *Bacillus anthracis* and that thymus desoxyribonucleic acid neutralized the antibacterial action of histone. Lecithin caused a delay in histone action, but could not halt it completely.

Thoma and Peterson (1950) found with *Clostridium sporogenes* that oleic acid plus "tween 40" will replace biotin and that either alone or in the presence of biotin causes an inhibition. In the absence of arginine, DL-ornithine is without effect, but with a small amount of arginine and a large amount of tyrosine the ornithine promotes growth.

A portion of Snell's (1949) review is so directly applicable to the problem at hand that it is here quoted.

> The extent to which such antagonistic relationships contribute to the nutritional requirements of organisms as determined under ordinary laboratory conditions is not always appreciated. A striking example is provided by the work of Washburn and Niven (1948) with *Streptococcus bovis*. This organism grew well in a medium containing appropriate salts, sugar, vitamins and L-arginine. Single additions to this medium of isoleucine, leucine, threonine, norleucine, or alanine completely prevented growth. Further single additions of valine, glutamic acid, cystine, or methionine in appropriate amounts fully restored growth to the original levels. The amino acid "requirements" of this organism for growth quite obviously depend upon the basal medium in which the requirement is determined; depending upon the conditions the organism appears to require none, or any one of several, amino acids. A mixture of phenylalanine and tyrosine, but neither one singly, was inhibitory to this organism; this inhibition was overcome by tryptophane. Thus an apparent requirement for a growth factor in a given medium does not necessarily mean that the organism lacks the ability to synthesize that substance. Additional examples of this type are the observations: (a) that the amount of serine required for growth of *L. casei* and *L. delbrueckii* depends upon the amount of threonine in the medium (Meinke and Holland, 1948); (b) that the amount of glutamic acid required by *L. arabinosus* for growth depends upon the aspartic acid content of the medium (Brickson et al, 1948); (c) that under certain conditions phenylalanine is required for growth of *E. coli* if tryosine is present in the medium, although the organism grows well if neither amino acid is present (Beerstecker and Shive, 1947); (d) that some yeasts grow well in the absence of thiamine, but addition of this vitamin inhibits growth and this inhibition of growth is alleviated by addition of vitamin B_6 (Schultz and Atkin, 1947). In each of these cases, an imbalanced nutrient solution has resulted in a qualitative or quantitative change in the requirement for additional nu-

trients. Similarly, the toxic effect of citrate for various lactic acid bacteria reported by Campbell and Gunsalus (1944) was found by MacLeod and Snell (1947) to result from a deficiency of manganous and magnesium ions induced in many of these organisms by the complex-forming action of citrate. Although a direct requirement for magnesium ion by *L. casei* could not be shown, this ion was essential for growth in the presence of citrate.

In addition to what was quoted from Snell the results of Washburn and Niven (1948), reported in their table 3, are very pertinent. The effects of varying concentrations of phenylalanine and tyrosine upon the growth of *Streptococcus bovis* are given. When present in a 1:1 ratio, these substances caused complete inhibition and, as they varied from this proportion, inhibition progressively decreased. This held true for concentrations from 5 mg. per 10 ml. down to 0.2 mg.

Lewis (1952) has found, in the study of *Alternaria solani*, a parasite of tomatoes, responses similar to some of those noted above. This fungus was found to grow on inorganic foods plus glucose, but its lag and acceleration phases were long. When biotin, choline, and thiamine were present, these phases were short. Many inhibitions and reliefs of inhibitions have been observed. When 22 amino acids at certain concentrations were added singly to the medium containing the three vitamins, the following effects were noted (Lewis, unpublished). Aspartic and glutamic acids increased growth, while beta-alanine and cystine strongly inhibited growth. Hydroxyproline, norleucine, tryptophane, tyrosine, valine, and norvaline inhibited to a lesser extent. The others had small or varying effects. Inhibition by beta-alanine was overcome by each of the 22 amino acids except arginine, histidine, hydroxyproline, lysine, ornithine, tryptophane, and tyrosine.

A second fungus, *Claviceps purpurea*, the cause of ergot of rye, has also been studied (Lewis, unpublished). In addition to inorganic foods and glucose its essential foods are biotin and glutamate. Beta-alanine enhances growth on this medium when used in concentrations that cause strong inhibition of *Alternaria solani*. Alpha amino n-butyric acid partially inhibited growth. Numerous other inhibitory and apparent foods have been noted, but are still to be further characterized.

For the parasites noted above we find substances that enhance and substances that inhibit the activities of the parasites. We find also that enhancement or inhibition depends upon the nutritional environment in which the substances operate. Is it not readily conceivable that these or similar factors can determine the success or failure of a parasite in its host?

Dubos (1950) has answered this question affirmatively in relation to the tubercle bacillus.

> Although little is understood of the mechanisms whereby organic acids affect the growth of tubercle bacilli, the known facts make it likely that the findings *in vitro* may be of some significance in analyzing the factors which determine the fate of tubercle bacilli *in vivo*. Some of the organic acids—the keto and dicarboxylic acids—seem to act only as growth-promoting agents and never as inhibitors of tubercle bacilli (at least in the range of physiological concentrations). Others—like lactic acid, propionic acid, butryic acid, capric acid, oleic acid, lignoceric

acid, etc.—are markedly growth-promoting in low concentrations (particularly near neutrality) and inhibitory in higher concentrations, particularly at more acidic reactions. All these substances are normally produced, either in the course of tissue metabolism, or by autolytic changes following necrosis, and some of them occur *in vivo* in the range of the concentrations which have been found to inhibit bacillary growth in the presence of serum. It seems legitimate, therefore, to postulate that they are not without influence on the evolution of the tuberculous lesion.

Alton and Orth (in Gaumann, 1950) have correlated, in part, the nutrition of a parasite with the substances present in the host. They found that *Phytophthora infestans*, a fungus parasite of potato, readily utilizes most of the amino acids as foods, but arginine is strongly inhibitory. When the nutrition of the host is modified so as to increase its non-protein nitrogen content without increasing its arginine content, the host becomes more susceptible. When the arginine content of the host increases and the non-protein nitrogen remains constant, the host becomes more resistant. When both increase there is no change in susceptibility. Thus arginine alone is not the determining factor; rather it is the balance between arginine and the other factors that determines the host's resistance or susceptibility.

The work of Page, Goodlow and Braun (1951) has demonstrated that *in vitro* inhibitions can also be active *in vivo*. Strain S° of *Salmonella typhimurium* was less virulent to guinea pigs and more susceptible to threonine inhibition *in vitro*. Strain S^{t} was more virulent and less susceptible to threonine. When S° and S^{t} were inoculated as a mixture into guinea pigs, only 4 out of 20 animals died within four days and the population recovered from the animals was primarily S°. When the same mixture of S° and S^{t} was used and the animals were simultaneously injected with threonine, 10 out of 20 animals died within four days and the population recovered consisted of a much higher proportion of S^{t}, the strain with greater virulence and greater *in vitro* resistance to threonine.

These facts add considerable support to the assumption that *in vitro* nutrition can be correlated with *in vivo* conditions provided sufficient details of the parasite's complex nutrition, which includes both inhibitory and promoting factors, can be worked out.

In view of the above facts on nutrition it is clear that one must always consider the nutritional environment in assessing the effects of a nutrilite. If this is kept in mind, a common type of fallacious reasoning about the parasitic relationship is overcome. The reasoning is thus: "The parasite grows on a simple synthetic medium; all potential hosts supply the ingredients of this simple medium; some hosts are resistant and some susceptible; therefore nutrition cannot account for species and varietal resistance and susceptibility." This reasoning cannot be right because the host supplies a complex environment and, when applied to this environment, the facts concerning a minimal or a simple synthetic medium are of little value.

LINES OF INVESTIGATION SUGGESTED BY THE HYPOTHESIS

The balance hypothesis should be of value in guiding further experimentation even though it be received with skepticism on other counts. It sug-

gests: (1) investigations into the complex nutritions of parasites that can be made to grow on synthetic media; (2) a comparison of portions of the complex nutritions of the biologic forms of a parasite species; (3) a comparison of portions of the complex nutritions of a spectrum of parasites extending from those with a wide host range at one end of the spectrum to those with a narrow range at the other; (4) studies on the limits of variability in relation to nutrition that exist in a parasite species as found in nature or as its limits can be modified in the laboratory.

Once the nutrition of a parasite begins to be known in terms of promoting and inhibiting substances, the balance hypothesis suggests: (1) the kind of analyses that should be run on the host in an attempt to locate significant factors in the definitive nutrition of the parasite; (2) a reexamination of portions of the parasite's complex nutrition in light of factors present in the host; (3) a continuation of these trial and error approximations in nutrition studies and analyses until the definitive nutrition, or nutritions, of the parasite is determined; (4) an examination in like manner of the problem of elective localization; (5) experiments in nutrition therapy; (6) experiments on the effect of parasite-generated toxins or localizing substances in order to determine shifts in the balance of the host's metabolites that are available to the parasite; (7) that in some cases virulence may be explained in terms of normal host metabolites acting on the parasite; (8) that, when antigen-antibody or phagocyte immunity is absent, changes in resistance and susceptibility due to age or changed nutrition of the host can be explained in terms of a changed metabolism of the host.

DIFFICULTIES OF THE HYPOTHESIS

The balance hypothesis poses some difficulties that cannot well be evaluated at present. How complex will the definitive nutrition of parasites prove to be? Will inorganic nutrients be critical in their effects? Will it be possible to develop a theory of nutrition, or perhaps a theory of ecology, which will permit a simplification of these aspects of the problem? If no simplifying concepts are developed, which combinations of promoting and inhibiting substances that are worked out *in vitro* should one look for in the host? Will present knowledge of metabolites be sufficient to produce correlations or can they be expected only after further progress in biochemistry? We know that peptides are important as growth promoters and inhibitors (Foster, 1949; Simmons and Fruton, 1951); will they become critical factors in determining resistance and susceptibility? Will the absence of the host proteins from the *in vitro* studies invalidate the nutrition experiments?

Which metabolites in the host are available to the parasite? Can there be an active absorption of certain parasite metabolites by the host so that the parasite is unable to grow? How important are permeability factors? Are substances absorbed by the parasite by simple diffusion or by active absorption? How does a parasite secure its foods from tissues where the foods do not exist in a free state, but only instantaneously in a metabolic stream? What is the relation of the parasites' digestive activities to the

problem? Can one distinguish the effects that are due to the metabolite balance from those that are due to antibodies, phagocytes, capsules, antibiotic substances, structural resistance, etc.?

This last question brings up the matter of the position of the balance hypothesis in relation to the established theories of resistance and susceptibility. It would seem at present that in all parasitic relationships the parasite is under the influence of the growth-promoting and growth-inhibiting substances that are the metabolites of the host. In most cases the metabolites are the critical factors in the relationship but in special cases known factors other than metabolites, as here considered, come into play and the extent of their effect may be minor, partial, or major in determining the outcome of the relationship. The balance hypothesis in no way attempts to displace established theory; it is simply an attempt to add to the existing theoretical structure a background sufficiently fundamental that it will be useful in studying parasitism in both the plant and animal kingdoms.

SUMMARY

It is hypothesized that large classes of facts concerning host-parasite relationships in both the animal and plant kingdoms can be understood in terms of the biochemistry of the host and the nutrition of the parasite without recourse to special agents such as antibodies, antibiotic substances, phagocytes, barriers, enzymes, and toxins. The hypothesis is made reasonable by what is known of the positive and negative effects of various nutrilites upon the growth of organisms and by what is known about the presence of these same substances within host organisms. Some facts which give circumstantial support and a few facts which give direct support to the idea are presented. Mention is made of possible lines of investigation to be followed and of possible difficulties to be encountered.

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POLYMORPHISM, LINKAGE AND THE BLOOD GROUPS

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POLYMORPHISM AND LINKAGE

Haldane (1930), when considering the evolution of dominance, pointed out that in the grouse locusts *Apotettix eurycephalus* and *Paratettix texanus* and the fish *Lebistes reticulatus* "mutant genes" for color-pattern are dominant and not recessive and that there are far fewer linkage groups than chromosomes. He suggested that translocations and duplications might be responsible for this association between the dominance of the "mutants" and linkage. He also maintained that the facts were not in agreement with Fisher's (1928) theory of dominance. However, Fisher (1930a) showed that the data were not inconsistent with his hypothesis and pointed out that the land snails *Cepaea nemoralis* and *Cepaea hortensis* could be added to the list of highly polymorphic animals with a "universal recessive" and with linkage between the genes responsible for the polymorphism. He showed that in the breeding cage the heterozygotes in *A. eurycephalus* were at an advantage of about 7 per cent to the dominant homozygotes. Later (1939) he found that the heterozygotes were at a 5 or 6 per cent advantage to the "universal recessive." From random samples of *P. texanus* taken in the wild he was able to show that (with one exception) individuals heterozygous for two dominants were at a disadvantage of about 40 per cent to those carrying a single dominant. Consequently in the wild, chromosomes carrying two dominants are extremely rare or absent. Moreover, when the *A. eurycephalus* data were taken into consideration it appeared that the very great disadvantage of double dominants was not an effect of viability that could be found in the breeding cage and must therefore be caused by ecological factors operating in the wild. It appeared that the genes, advantageous by themselves, interacted with one another in such a way that in combination they were very disadvantageous. As would be expected, there was good evidence that the selective values of the various genotypes varied from population to population.

Nabours, Larson and Hartwig (1933) have investigated the genetics of the color-pattern polymorphism in the grouse locust *Acrydium arenosum*. In this species linkage is not so close as it is in *P. texanus* and more linkage groups are involved. Genes having analogous effects on the color-pattern can be found in *P. texanus*, *A. eurycephalus* and *A. arenosum* and they have different linkage relationships in the three species. Mather (1951, p. 97) has pointed out that in Nabour's data on *A. arenosum* there is very good evidence for differences in viability between some genotypes, and here again it is due to an interaction between loci (that is to say the presence of one gene alters the selective value of another at a different locus). In

one case at least there is a deficiency of the double dominant class and a greater shortage of one of the two single heterozygous classes. Such differences in viability indicate quite clearly that selection is large and therefore that the polymorphism found in this species must be maintained by it and not by genetic drift.

In *Tettigidea parvipennis* there are ten color forms that behave as multiple alleles and six others which are not apparently linked to them (Bellamy, 1917; Good, 1941; Nabours, 1929). In *Telmetettix aztecus* there are at least four varieties which also behave as multiple alleles and are dominant to the most common form in the wild (Nabours and Snyder, 1928). However, the relative viability of the color forms in *T. parvipennis* and *T. aztecus*, does not seem to have been investigated. Other species of grasshoppers also show extreme polymorphism particularly in the genus *Chorthippus* (Rubtzov, 1935). Rubtzov (1935) showed that parallel variations were found in 13 species of Chorthippus (including *C. albomarginatus*, *C. parallelus*, *C. bicolor* and *C. longicornis*) and in 13 species from various other genera. He found that the appearance of the varieties could be altered by the environment in *C. albomarginatus* but that they could not be converted into each other, and concluded that they were genetically controlled. He suggested that there were marked differences in the behavior of the varieties and showed that their relative frequency changed with time. Also their frequency was correlated with the nature of the environment. *C. parallelus* has been investigated genetically (Sansome and La Cour, 1935). Fourteen genes were definitely identified and there were indications that other major genes were segregating. In only two cases was strong linkage found. Sansome and La Cour also showed that the frequency of the genes varies from population to population and stated that "parallel variation in type between *C. parallelus* and *Stenobothrus lineatus* in one habitat has been frequently noticed." In *C. longicornis* Creighton and Robertson (1941) found that the four forms they investigated behaved as multiple alleles. It therefore appears that in the genus Chorthippus polymorphism is genetically controlled, that the number of groups of linked genes varies from species to species, that the polymorphism is maintained by selection and that the selective values and consequently the frequency of genes varies from population to population.

In the grasshoppers the genes responsible for the polymorphism appear not to be sex-linked, but in the fish *Lebistes reticulatus*, as Fisher (1930a) has noted, not only are many genes from the wild found on the sex-chromosomes but also they are nearly always confined to the Y-chromosome when first investigated genetically, although they can cross over to the X-chromosome in breeding experiments. Fisher has suggested that the rarity of these partially sex-linked genes on the X-chromosome is due to the mutants being advantageous in the male but disadvantageous in the female.

Recently Haskins and Haskins (1951) have made a study of *L. reticulatus* in the wild. They did not manage to obtain enough data to show if the genes which could cross over from the X to the Y-chromosome were commoner in

the Y or not, but more extensive studies should settle this point. They did not find the autosomal recessives gold, blonde and albino in the wild. Moreover, all the wild color genes were sex-controlled, which suggests that many of the mutants that are or become sex-controlled are retained in the population by selection whereas other color genes are eliminated.

Gordon (1947a) also investigated sex-linked genes in the fish *Platypoecilus maculatus* but unfortunately did not say if "dominant" genes are commoner in the X or the Y-chromosomes in the wild. Autosomal "multiple alleles" have also been found (Gordon, 1947b). The published results indicate a difference in sex ratio in the recessive class as compared with those carrying one or two dominants, a point that clearly needs investigating as the genes are not sex-linked. Gordon and Gordon (1950) obtained discrepancies in the expected and observed frequency of certain combinations of genes in the wild. This suggests that there is strong selection as a result of an interaction between genes.

Fisher and Diver (1934) showed that in *Cepaea nemoralis* the gene for pink shell and that for unbanded are linked, and that the crossover value varied. Diver (1932) stated that all the genes affecting shell color and banding observed by him appeared to be linked in this species. Cain and Sheppard (in manuscript) have found evidence for linkage between genes for pink, unbanded and non-pigmentation of the bands (albino). The brown form also shows an association with unbanded and may be an allele of pink or linked to it. As in *P. texanus*, chromosomes carrying certain combinations of genes may be absent or nearly absent in some colonies and the gene frequencies vary from population to population. Diver (1940) has stated that these differences in gene frequency are due to genetic drift. Lamotte (1951, 1952) has also claimed that variations in the frequency of the gene for unbanded are controlled by genetic drift, and that the polymorphism is maintained by an unusually high mutation rate and reverse mutation rate of the order of 1×10^{-4} and 5×10^{-4} respectively. However, Cain and Sheppard (1950 and 1952) and Sheppard (1951, 1952) have shown that selection through visual predation for color and banding determines the frequency of the varieties in each colony. More recently (Cain and Sheppard in manuscript) they have shown that Lamotte's data are in agreement with their own although his conclusions are not, and that there must be non-visual selection for certain gene combinations to maintain the polymorphism.

They did not specifically investigate *C. hortensis* but did obtain some data which suggest the action of selection. However, Schnetter (1950) has shown strong selection. The absence of certain gene combinations in particular colonies of *C. hortensis* indicates that the genes for pink and unbanded are also linked in this species. The more frequent absence of at least one of the crossover classes in colonies of *C. hortensis* as compared with *C. nemoralis* suggests that either linkage is closer than in *C. nemoralis* or that selection is greater. It is also interesting to note that yellow and unbanded tend to be commoner in *C. hortensis* than in *C. nemoralis* where both pink banded and unbanded are very common on some backgrounds.

In the ladybirds polymorphism is common and the differences between the varieties are often due to "multiple alleles" (see Shull, 1943). In *Harmonia axyridis* a large series of multiple alleles are known and there are differences in the frequency of the varieties between populations. Moreover, not only do the populations vary depending on the plant on which they are living, but their frequencies also alter with time (Komai, Chino and Hosino, 1950; Komai and Hosino, 1951; and Tan, 1949). It has also been shown that there is a difference in the viability of some of the forms during hibernation, indicating that there is strong selection acting. *Adalia bipunctata* also has a number of forms controlled by what appear to be multiple alleles (Shull, 1943). Timoféef-Ressovsky (1940) has shown that the frequency of these forms fluctuate violently from season to season and year to year as the result of very strong selection.

In another beetle, *Phytodecta variabilis*, de Zulueta (1925) has found a situation similar to that in *Lebistes reticulatus* except that the genes are not sex-controlled. He found that the animal was polymorphic and that there were at least four distinct forms which varied in their frequency from place to place. Moreover, the frequency of the varieties was not the same in the two sexes, insects with lines on the elytra being rarer in the males. Greenish yellow and red elytra were commoner in the males than in the females, but there was no detectable difference between the sexes as far as the black forms were concerned. De Zulueta showed that the genes responsible for the polymorphism were on the sex-chromosomes and could be found on both X and Y. The difference in the frequency of the genes on the X and Y-chromosomes suggests very strongly that selection is acting and that there is an interaction with sex. That is to say, that the selective value of the genes is not the same with respect to their alleles in the two sexes as has been suggested also for *L. reticulatus* (Fisher, 1930a). Bateson (1895) and Doncaster (1905) have reported a few beetles with lines on the elytra and with a red ground color, whereas de Zulueta always found yellowish green elytra associated with stripes. These red forms might well be due to recombination as the result of crossing over and if this be true it means that the genes are not alleles, which would be expected if the situation is analogous to that found in fish.

From the foregoing discussion it appears that most of those polymorphic species which have been adequately investigated have a large number of characteristics in common. There is frequently a "universal recessive" and a number of dominants which do not always mask one another's effect. There is often strong linkage between many of the genes, ranging in degree from so little crossing-over that they behave as multiple alleles up to crossover values of the order of 20 per cent. In one species linkage is almost absent (*C. parallelus*) but is very close in a related species (*C. longicornis*). The polymorphism is usually found in many related species and in *C. nemoralis* and *C. hortensis* it is shown to be very old by the fossil record. Finally selective values associated with polymorphism fluctuate from population to population and are often so large that the action of selection can

be demonstrated easily. Values of the order of 40 per cent are found, and those of the order of one per cent must be very common.

THE BLOOD GROUPS

Although only morphological characters have been considered so far, it is of interest to note that the blood groups in man fit into the picture very well. However, because of the small quantity of data available on the rarer groups only the common ones will be considered here.

The A, B, O system behaves as a series of multiple alleles as do many characters in *P. texanus* and other animals (see above). Moreover, the frequency of the alleles varies from population to population. Wright (1940) and others have suggested that the different A, B, O groups are neutral in selective value and that variations in gene frequency are due to genetic drift. However, Ford (1945) has suggested that the stability of the ratios, even when the populations have moved from their place of origin a very long time ago, shows that the gene frequencies are determined through selection by the gene complexes of the individuals in the population. There is now good evidence that selection is acting. Race and Sanger (1950) point out that several workers have found a difference in the proportions of A and O children when the mother is O and the father A compared with those found when the father is O and the mother A. This difference, if it is substantiated, suggests that some factor, perhaps haemolytic disease, is affecting the ratio and that selection is operating. Moreover, they give good evidence that the mothers of children with haemolytic disease due to anti-Rh are more often compatibly mated on the A, B, O system (in the sense that husbands can give them blood) than are a group of unrelated women. It is unlikely that this interaction between the A, B, O and Rh blood groups has no effects on the selective value of the genes concerned. There is also some evidence that the sex ratio is affected by these genes (Sanghvi, 1951), as well as cancer of the stomach giving slight selection against group A (Aird, Bentall and Fraser Roberts, 1953). If this sex-ratio effect is substantiated then alterations in the gene complex affecting the sex ratio will also alter the selective value of the A, B, O genes. This supports Ford's (1945) view that the gene complex controls the frequency of these genes in the population. Finally as in the other instances of polymorphism that have been considered, this blood group variation is apparently also found in related species (the anthropoid apes).

The M, N, S, s, Hunter, Henshaw group is very similar to the previous one except that here it is more likely, though not proved, that very closely linked genes rather than multiple alleles are concerned. The frequency of the genes and combinations again vary from population to population. Many workers' data show an excess of MN offspring from M N × M N matings (see Race and Sanger, 1950). This discrepancy has in the past been attributed to a faulty technique in identifying the M N group. However, this does not explain the discrepancy adequately as it is only found in family data and not in random samples. Moreover, the data of Race and Sanger

(1950, p. 45) cannot suffer from this defect in technique as the typing of random samples and families was done with the same sera over the same period. Nevertheless they still found an excess of M N children in the family data although it was not quite significant ($\chi^2_{[1]} = 3.05.$). However, it was in the same direction as that found in other workers' results and it is therefore almost certainly a real effect and must be taken as evidence of selection in favor of heterozygotes.

The Rh blood groups are again similar in these respects to the ABO and MNSs groups. The frequency of the genes varies from population to population, and although there is disagreement, the weight of evidence seems to be in favor of closely linked genes rather than an allelic series (see Race and Sanger, 1950). In the Rh blood groups it is clear that very high selective values are involved as a result of haemolytic disease. Haldane (1942) has maintained that the polymorphism must be transient (as defined by Ford, 1940) because of the selection. However, Fisher and others (see Race and Sanger, 1950) have suggested that the loss of children through haemolytic disease may be over-compensated by an increase in the number of children born, thus maintaining the polymorphism. The fact that human populations can also be polymorphic for Kell which is known to cause haemolytic disease and for the A B O group which is strongly suspected of causing it, suggests that the polymorphism is not transient but stable.

The only other blood group in man which need concern us here is Lewis. The gene is probably not allelic with, but linked to another (the secretor gene) whose only known effect is not strictly that of a blood group gene but determines the presence or absence of the A B or H substances in saliva and other body fluids. This seems to be an example in the human blood groups of linkage associated with polymorphism where the linked genes do not apparently control similar characters.

In cattle, blood group differences similar to those of the human Rh system have been found (Stormont, Owen and Irwin, 1951; Stormont, 1952). These can be explained by a very extended series of multiple alleles (at least 80 at one locus) or a small number of linked genes. Similar but smaller systems have been found in ducks and chickens (McGibbon, 1945; and Briles, McGibbon and Irwin, 1950). Unfortunately wild populations of none of these animals nor their close relatives have been studied. Consequently there is no evidence for the presence of strong selection or polymorphism under natural conditions although there is for chickens under domestication (Schultz and Briles, 1953). The similarity between these systems and human blood groups suggests that they have all arisen in the same way.

It is clear from the evidence that the blood group polymorphism is comparable with the morphological polymorphism in fish, grasshoppers, beetles and snails in every detail except that there seem to be rather more linkage groups in man than in most other species considered. It is possible that a few of the highly polymorphic mimetic butterflies may also come into this category. Although this is not known, Komai (1953) has shown polymorphism

in a group of non-mimetic butterflies, and found evidence of linkage and selection against a gene combination in one of these species.

EVOLUTION OF LINKED SYSTEMS OF GENES

The very common occurrence together of extreme polymorphism (often with a "universal recessive"), close linkage, and high selective values associated with an interaction (genes having very different selective values in different combinations) between factors suggest that these phenomena are causally connected. It seems worthwhile to consider how this situation has been evolved, provided it is remembered that any conclusions can only be in the nature of an hypothesis to be investigated experimentally.

One type of polymorphism that has been extensively investigated and seems common is characterized by chromosome rearrangements. Some species of Sciara often show minute duplications and deficiencies as well as inversions (White, 1945). Grasshoppers often have chromosome abnormalities which are either inversions or shifts (White, 1945, 1951) and populations of Chironomus (Philip, 1942) and Drosophila (Dobzhansky, 1951) are often polymorphic for inversions and in many species one or a few of the chromosomes are more variable than the others. Inversions in several species of Drosophila are found to have very high selective values (Dobzhansky, 1951) and exist in a state of stable polymorphism.

If a new advantageous mutant arises in one of these structural arrangements it might well spread but be unable to replace its allele in the population because of the chromosome polymorphism. In this way inversions and other rearrangements might well act as a "net" which catches advantageous genes and stops them reaching fixation in the population. If this happened, a number of linked advantageous genes would accumulate, some in coupling and some in repulsion, and result in a number of groups with linkage within them. It is interesting to note that in the grasshoppers, where polymorphism for linked genes is common, several types of chromosome at varying frequencies have been found. Harman (1920) has claimed that one of the dominant genes found in Paratettix is actually associated with a chromosome abnormality, although she did not give sufficient data in her paper to prove this. Inversions have been reported in the two polymorphic species *Chorthippus parallelus* and *C. bicolor* by Darlington (1936). Fisher and Diver (1934) found differences in crossover values between color and banding in Cepaea which suggests but does not prove the presence of inversions; and Koller (1937) has found what was apparently an inversion in man. It therefore seems possible that chromosome rearrangements are sometimes associated with extreme polymorphism and linkage as Haldane (1930) suggested. However, there are at least two reasons why mere inversions as such are unlikely to be responsible for maintaining the polymorphism.

(1) The inversions would not necessarily affect only genes controlling the same type of character (such as pigment formation and localization) but would also prevent all types of advantageous genes from reaching fixation whereas the linked genes seem usually to affect only one type of character

in any one species. Thus in plants, (for example, Primula) the linked genes may affect several characters, but they are apparently all concerned with outbreeding. This may of course be due to the fact that only one type of character will be recognized because of the method of observing it. For example, blood techniques would not show up morphological characters although they would demonstrate the presence of the secretor gene which, as pointed out above, is an exception in that it controls a different character.

(2) There seems no very obvious reason for believing that any mutant appearing in one inversion will not in time appear in an uninverted chromosome and therefore be able to replace its allele in the population. Only those genes advantageous in one inversion but not in the other arrangement will remain polymorphic.

If a particular mutant *A* appeared which was disadvantageous in most gene complexes but advantageous in the presence of gene *B* at another locus then it might spread if it were linked to *B*. However, it would probably replace its allele (*a*) in the population unless *B* were unable to replace its allele (*b*). Fisher (1930b) has pointed out that if there are two pairs of alleles in a population, which control contrasting characters *C-c* and *D-d* respectively, then if *C* is at an advantage to *c* in the presence of *D* but disadvantageous in combination with *d, and D* is advantageous with *C* but not with *c*, stable polymorphism can result. Moreover, if the genes are on the same chromosome, linkage between the two loci will increase as a result of selection. This would account for the close linkage or apparent multiple allelism in the blood groups and similar polymorphic systems, particularly as genes controlling the same type of character seem more likely to alter radically the selective value of each other than do those affecting different characters. Occasionally genes controlling different characters may interact strongly. The gene for Lewis and that for Secretor may be a case in point and also the genes for color in *Apotettix eurycephalus* and the lethal which increases viability when heterozygous. There is no reason to suppose that the same genes have the same interactions in different environments or gene complexes. Consequently genes will be found in different proportions from population to population and the combinations that are at an advantage, and are therefore common, will vary also.

The hypothesis that linkage will increase between genes which alter each others' selective value, when they are maintained in a state of polymorphism, seems to account for the close linkage in the human blood groups but does not account for the situation in the grasshoppers so adequately. In some grouse locusts a very high proportion of the genes are on one chromosome and in a single close linkage group. This could be accounted for if they arose as a result of duplications and subsequent mutation. However, in *P. texanus* and *A. eurycephalus* it would require a large number of duplications to account for all the linked genes. Moreover, in some species there seems to be little linkage at all (*C. parallelus*) although in a closely related species (*C. longicornis*) it is very strong.

It is probable that very closely related species often differ by translocations. In most animals differences between species and sub-species due

to translocations have not been adequately studied, but in Drosophila where such a comparison is relatively easy many translocation differences have been found (see Dobzhansky, 1951). Moreover, in Triturus similar changes between sub-species were found directly they were looked for using a suitable technique (Callan and Spurway, 1951); and Callan (personal communication) has found them between species in this genus. White (1945) has suggested that chromosome variation between some species of grasshoppers is best explained by translocations. Consequently it seems not unreasonable to suppose that such differences are fairly common and it has often been pointed out when they do become established in a population they will tend to cause speciation as the result of selection for sexual isolation. Any translocation which brought two or more interacting genes on to the same chromosome would increase the linkage between them and consequently be at an advantage in this respect. Such translocations will on the average have a better chance of survival than many others. Over long periods of time nearly all the interacting genes might accumulate on one chromosome, particularly if very large selective values due to interactions are involved. For example, in *P. texanus*, some of the selective disadvantages are as high as 40 per cent and there is a very large linkage group. In other species the process might proceed very much more slowly or not at all so that varying numbers of linkage groups will be found in them. Once two interacting genes are on the same chromosome, inversions and shifts within the chromosome or the suppression of chiasma formation in some regions will also increase the linkage.

Cepaea nemoralis is not very mobile (Lamotte, 1951) so that colonies of it may often be formed from one or a few individuals and there may be quite a number of colonies dying out or being established at any one time. Now in this species certain combinations are at an advantage in some environments and at a disadvantage in others (pink unbanded is advantageous in woods and disadvantageous in smooth green surroundings). If a woodland colony is started from one living on short turf then the crossover class will be at an advantage and there will be selection against linkage until the crossover class becomes frequent. Consequently, differences in crossover value might be expected in this species and they are apparently found (Fisher and Diver, 1934). *Lebistes reticulatus* seems to show a particular case of interaction where the "dominant" genes are at a disadvantage in the female, but at an advantage in the male (Fisher, 1930a); that is to say, there is an interaction with sex. Consequently the genes may tend to be confined to the Y chromosome in nature. It would be interesting to know how the different genes affect one another in the wild. It is clear that many of the sets of genes behaving as multiple allelic series are really closely linked genes, but this does not preclude the possibility that there are several polymorphic situations where a real allelic series is responsible for the variation. The hypothesis that systems of closely linked genes have been evolved, as the result of selection for linkage, does not explain situations where there is no polymorphism and the mutants are rare recessives which are seldom found in the wild. However, if a polymorphic species evolved a group of linked

genes and then, as the result of a change in selection pressure, became monomorphic, any recessive mutants arising at the loci that had been involved in the polymorphism would be linked and would probably affect the same characters.

The hypothesis that genes which alter each other's selective value, when maintained in a polymorphic state, will tend to accumulate on the same chromosome as the result of translocations and that linkage between them will then continue to increase as the result of selection, seems to account for the evolution of a system in which considerable polymorphism, close linkage and high selective values are associated. It also explains some of the differences in degree of these phenomena found between closely related species. It is possible that an investigation of the blood groups will throw considerable light on the subject. Moreover, the investigation of polymorphism in fish, snails, beetles and grasshoppers, all of which can be bred in the laboratory, may well help to explain the evolution and significance of the blood groups.

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SUMMARY

In a number of groups of animals extreme polymorphism is found and this is not usually confined to one species but is common to several related ones. Moreover, in a large proportion of these species more of the genes responsible for the polymorphism are closely linked than would be expected by chance. Many of these genes have been shown to be subject to strong selection and its direction and intensity often varies from combination to combination and population to population.

Although in insects, snails and fish the polymorphism is characterized chiefly by color-pattern differences, in mammals and birds blood groups are involved. The surprisingly frequent association between polymorphism, linkage and strong selection indicates that there is some causal connection between them and suggests that the linkage has been evolved.

It is known that if a species is polymorphic for two pairs of linked genes and if the genes have different selective values in different combinations linkage between them is likely to increase as the result of natural selection. It is suggested that if the genes are on different chromosomes any translocation which moves them on to the same one will increase the linkage between the genes and therefore be at an advantage in this respect at least. Consequently such translocations will, on the average, have a better chance of surviving than any others that may arise. In this way genes which alter each other's selective coefficient will tend to accumulate in one or a few linkage groups and the speed at which this happens will depend in part on

the strength of selection. The differences in the rate of the accumulation of linked genes accounts for the variation in the number of linkage groups found between closely related species.

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THE MUTAGENIC ACTIVITY OF DYES IN *DROSOPHILA MELANOGASTER*

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Since the discovery of the mutagenic activity of mustard gas in Drosophila (Auerbach and Robson, 1946), an ever-increasing amount of work has been published on the cytological and genetic effects of various chemicals. It is therefore not surprising that several attempts have been made to develop a general theory of the action of chemical mutagens, based on the assumptions that ultimately the processes leading to mutation are the same in all cases. Rapoport (1948), for example, has suggested that all mutagenic chemicals react with the protein part of the gene molecule rather than with the nucleic acid part, to bring about chemical changes such as alkylation or oxidation. Other workers have attempted to explain mutagenic effects in terms of the formation of cross-linkages between different parts of the protein molecules (Loveless and Revell, 1949). However, as Auerbach (1951) has pointed out, there does not seem to be any obvious reason why all mutagens should act in the same way. Reaction with either nucleic acid or protein moiety of the gene molecule would seem to offer at least two possibilities as far as the site of action is concerned. In order to test the first of these possibilities, the present investigation was undertaken with the express object of attempting to produce mutations in Drosophila by treatment with dyes known to combine under certain conditions with nucleic acids. Although some observations made a few years ago by Demerec and Luria (1945) gave little or no indication that dyes were likely to be mutagenic agents of especial interest, it was felt that Witkin's discovery (1947) of the mutagenic activity of pyronin in *Escherichia coli* justified a re-examination of the question in Drosophila.

METHODS

In all of the experiments reported in the present paper, the dye to be tested for mutagenic properties was included in the standard food medium at a concentration of about 0.1 per cent. Observations on the effects of injecting the dyes into larval stages or young adults will be presented in a subsequent paper dealing with a detailed analysis of the mutagenic activity of pyronin. With the exception of acridine orange, none of the dyes examined appeared to be particularly toxic.

The wild type stock of *D. melanogaster* used as a test object is one designated in this laboratory as +S50 iso. This stock originated from a single female collected near Melbourne in January, 1950, and has been rendered somewhat isogenic by strict brother-sister matings for fifty generations. The stock seems to have a low spontaneous mutation rate and is therefore well suited to this type of work.

Sex-linked recessive lethals were scored by mating treated males individually to three or four Muller-5 virgin females, offspring being collected only during the first ten days. The F_1 progeny from each male were aged en masse for about four days before the F_2 cultures were set up, enabling the number of cultures that failed to produce offspring to be kept down to about three per cent. All suspected lethals were subsequently confirmed by further tests.

MUTAGENIC ACTIVITY OF THE DYES

In a preliminary survey a number of small-scale tests were made in an attempt to eliminate those dyes in which mutagenic activity was either absent or so weak as to be of no particular interest. The results of this initial survey, summarized in table 1, suggest that the thiazine dyes, methylene blue and thionin, and the two members of the triphenylmethane series, crystal violet and methyl green, have little or no mutagenic activity. Phenosafranin and gallocyanin are of doubtful significance. Acridine orange is undoubtedly mutagenic, a result that was more or less to be expected, since the related compounds acriflavine and euflavine have been shown to possess mutagenic activity by Demerec (1949) and by Ephrussi and Hottinguer (1950).

TABLE 1

PRODUCTION OF SEX-LINKED RECESSIVE LETHALS BY THE ADDITION OF DYE TO THE STANDARD FOOD

Dye	No. of chromosomes tested	Lethals	Per cent lethals
Pyronin (ethyl)	490	5	1.02
Acridine Orange	791	5	0.63
Neutral Red	633	2	0.33
Gallocyanin	769	2	0.26
Phenosafranin	502	1	0.20
Methyl Green	1067	1	0.09
Crystal Violet	974	0	....
Thionin	748	0	....
Methylene Blue	813	0	....
Controls	1068	1	0.09

The failure of methylene blue to increase significantly the frequency of recessive lethals confirms observations made by Demerec and Luria (1945) incidental to the testing of the aerosol method for the administration of chemicals to Drosophila. Battaglia (1950) has tested several basic dyes for ability to produce chromosome breakage (the Allium test) and finds that only methylene blue and toluidine blue are effective. However, since they cause fragmentation without recombination of the broken ends, it is hardly surprising that they do not appear to possess mutagenic activity.

The most interesting fact revealed by this preliminary survey was that in Drosophila, as in *E. coli* (Witkin, 1947), pyronin is definitely a mutagenic agent. Further tests were therefore made on this dye using samples that

had been purified in the laboratory. One of these larger scale tests was carried out on a different sample of pyronin to that used in the preliminary survey. On this occasion, although a definite mutagenic effect was observed, it was not as striking as had been expected. Repetition of the experiments with the original pyronin revealed that there was a genuine difference between the two samples. On analysis, the first sample with the higher mutagenic activity proved to consist of the tetraethyl derivative (pyronin B), whereas the second and less active sample was the tetramethyl derivative (pyronin Y). This interesting result led to the testing of rhodamine, differing from tetraethyl pyronin chiefly in that an additional benzene ring is attached to the central carbon atom, and of acridine red which, in spite of its name, is in fact a lower homologue of pyronin, being the dimethyl derivative (figure 1). The results of these tests, together with a further extensive set of controls, are listed in table 2. These figures suggest that the difference in mutagenic activity between methyl and ethyl pyronin is probably significant. Acridine red is perhaps a doubtful case and is best regarded as an extremely weak mutagen. Rhodamine, on the other hand, appears to be definitely although weakly mutagenic, but the difference between it and methyl pyronin, while suggestive, is not conclusive.

These observed differences in mutagenic activity seemed to offer an opportunity of testing the suggestion that dyes may bring about genetic effects by reacting with nucleic acids. A few observations were therefore

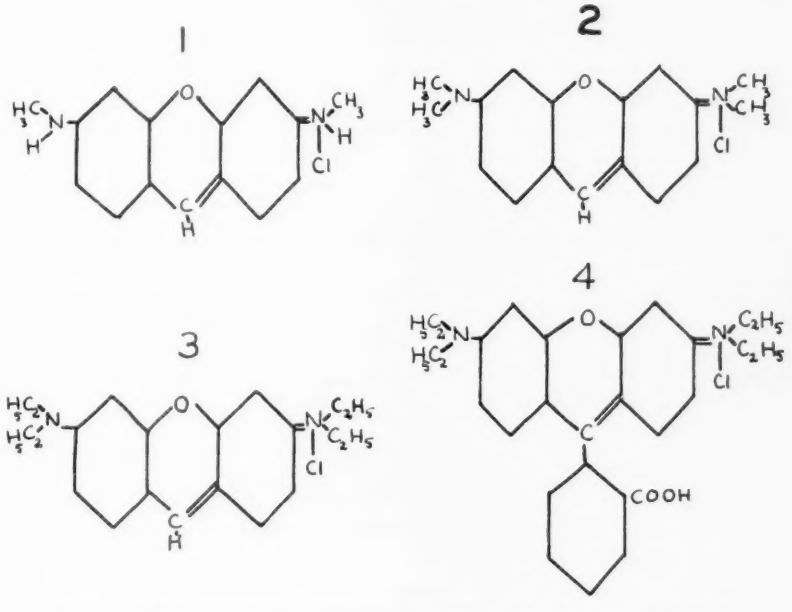

FIGURE 1: The structural formulae for (1) acridine red; (2) methyl pyronin; (3) ethyl pyronin; (4) rhodamine.

TABLE 2

MUTAGENIC ACTIVITY OF THE PYRONIN SERIES OF DYES

Dye	No. of chromosomes tested	Lethals	Per cent lethals
Ethyl pyronin	2005	24	1.20 ± 0.24
Methyl pyronin	1870	12	0.64 ± 0.18
Rhodamine	1882	7	0.37 ± 0.14
Acridine Red	1581	4	0.25 ± 0.12
Controls	3017	2	0.06 ± 0.04

made on the union in vitro of pyronin and its homologues with solutions of ribose nucleic acid.

THE UNION OF BASIC DYES WITH NUCLEIC ACID

The formation of a complex by the adsorption of a basic dye on to ribose nucleic acid tends to lower the solubility of the latter and may result in the formation of a precipitate. This fact can be utilized to demonstrate that dyes differ in their affinities for nucleic acid. By maintaining the dye concentration constant and varying the nucleic acid concentration, it is possible to determine the minimal amount of the acid required to form a precipitate in the presence of a particular dye. Results of such an experiment with yeast ribose nucleic acid are presented in table 3. It should be emphasized that in all cases where precipitates of RNA formed, the supernatant liquid still showed the typical color of the dye, indicating that it was present in excess. Precipitates with ethyl pyronin always formed at quite low concentrations of nucleic acid, whereas rhodamine and acridine red precipitated the acid only if the latter was at a relatively high concentration. Methyl pyronin occupies an intermediate position.

If a dye-nucleic acid precipitate is collected by filtration, it may be washed with absolute alcohol with only slow loss of adsorbed dye. However if the precipitate is treated with weaker alcohols, the dye may be washed out completely but at a rate depending on the nature of the dye.

TABLE 3

PRECIPITATION OF YEAST RIBOSE NUCLEIC ACID BY THE PYRONIN SERIES OF DYES

All solutions made up in M/150 acid phosphate. Tubes allowed to stand 48 hours at 15 C. Total volume = 10.0ml. Final dye concentration was 0.5×10^{-3} M in all cases.

No precipitate: −. Faint turbidity: +. Precipitate: ++. Copious precipitate: +++.

Dye	Nucleic Acid Concentration				
	0.0025%	0.005%	0.01%	0.03%	0.1%
Acridine Red	....	....	....	+	++
Rhodamine	....	....	....	+	++
Methyl pyronin	....	....	....	++	+++
Ethyl pyronin	++	++	+++	+++	+++

Whereas ethyl pyronin is washed out only very slowly, rhodamine and acridine red may be extracted quite rapidly, indicating that the ease with which the dye can be eluted from the adsorption complex is more or less inversely proportional to the ability of the dye to precipitate the nucleic acid. It is suggested that the differences are reflections of varying affinities shown by the dyes for RNA.

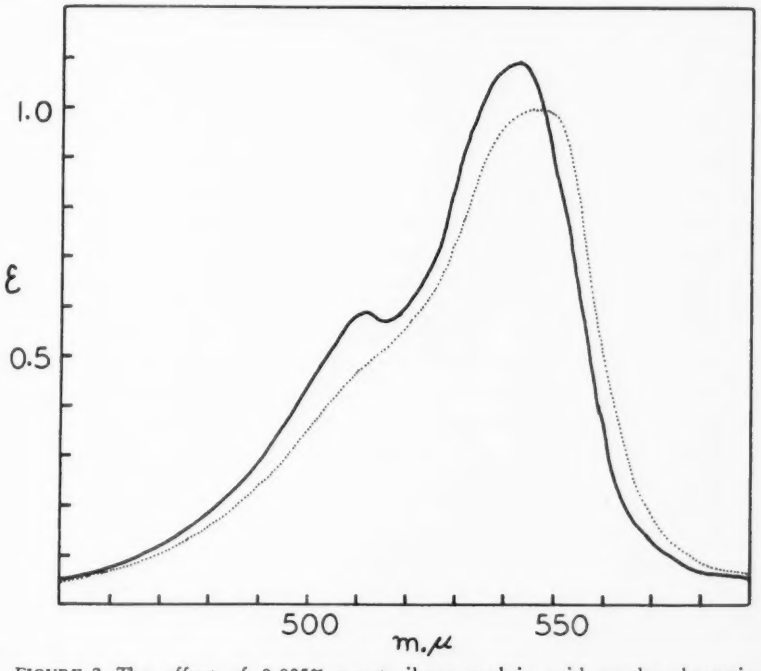

FIGURE 2. The effect of 0.005% yeast ribose nucleic acid on the absorption spectrum of 0.25×10^{-4}M methyl pyronin in 0.02M sodium acid phosphate, pH about 5.2. Full curve = dye alone. Dotted curve = dye + RNA.

While this general conclusion is probably justified, it seemed desirable to search for further evidence to substantiate it. A study was therefore made of the effects of nucleic acid on the absorption spectra of the dyes. In these experiments, the determination of the absorption spectrum was carried out immediately after the addition of the nucleic acid and before the formation of any precipitate had taken place.

Figure 2 shows the absorption spectrum of methyl pyronin in the visible region, as determined in the Beckman spectrophotometer. The curve shows two maxima, an alpha band at about 545 mμ and a beta band at about 510 mμ. These two bands are believed to be characteristic of single dye molecules and bimolecular aggregates respectively. Under a given set of physico-chemical conditions, there seems to be a definite equilibrium between the fraction of the dye present as paired aggregates and that present

as single, unassociated particles (Shepperd, 1942; Shepperd and Geddes, 1944).

As Michaelis (1947) has described, if a small amount of ribose nucleic acid is added to the dye, there is a shift in the absorption bands towards the red end of the spectrum. This is observable to the naked eye as a darkening of the dye solution and the development of a magenta color. There is also a considerable reduction in the extent to which the dye will fluoresce. Since there is also a suppression of the beta band, Michaelis suggests that the nucleic acid tends to prevent the formation of bimolecular aggregates of the dye. An alternative explanation for the spectral changes has been proposed by Kurnick (1950). He supposes that the effect is not the result of the suppression of dimer formation but is due to the orientation of the dye molecules as a result of attachment to an oriented structure, namely, the nucleic acid. This would account for Kurnick's observation that polymerized DNA is more effective in producing a given spectral shift than is RNA.

It will be noted that the two main effects of nucleic acid on the absorption spectrum, namely, the suppression of the maxima and the shifting of the entire absorption spectrum towards the red, act in the same direction in that they both will have as their result a decrease in the value of the extinction coefficient at the wave lengths of the maxima of the pure dye. Hence a convenient measure of the overall effect of nucleic acid on the absorption spectrum is obtained by calculating the product of the extinction coefficients at the wave lengths of the alpha and beta bands and plotting this as a function of the nucleic acid concentration. The appropriate wave lengths for the four dyes are indicated in table 4, while in figure 3 the products of the extinction coefficients are plotted against RNA concentration. The different values of these products for the pure dyes in the absence of any nucleic acid merely reflect the differences in the intensity of the absorption bands of the dyes themselves. The value of the product is reduced on addition of nucleic acid but tends to become constant as the concentration of nucleic acid rises. This point is probably reached when all the dye has been adsorbed. Since all the experiments were carried out under carefully controlled conditions using the same sample of RNA throughout, it seems reasonable to assume that the differences in the concentration of RNA required to depress the product of the two coefficients to a constant value are reflections of different affinities of the dyes for nucleic acid. Since ethyl pyronin appears to combine most readily with nucleic acid and since it also shows a greater mutagenic activity than do the other three, it is suggested tentatively that the mutagenic activity

TABLE 4

ABSORPTION MAXIMA OF THE PYRONIN DYES

	Acridine red	Rhodamine	Methyl pyronin	Ethyl pyronin
Alpha band	540 mμ	550 mμ	545 mμ	550 mμ
Beta band	510 mμ	522 mμ	510 mμ	512 mμ

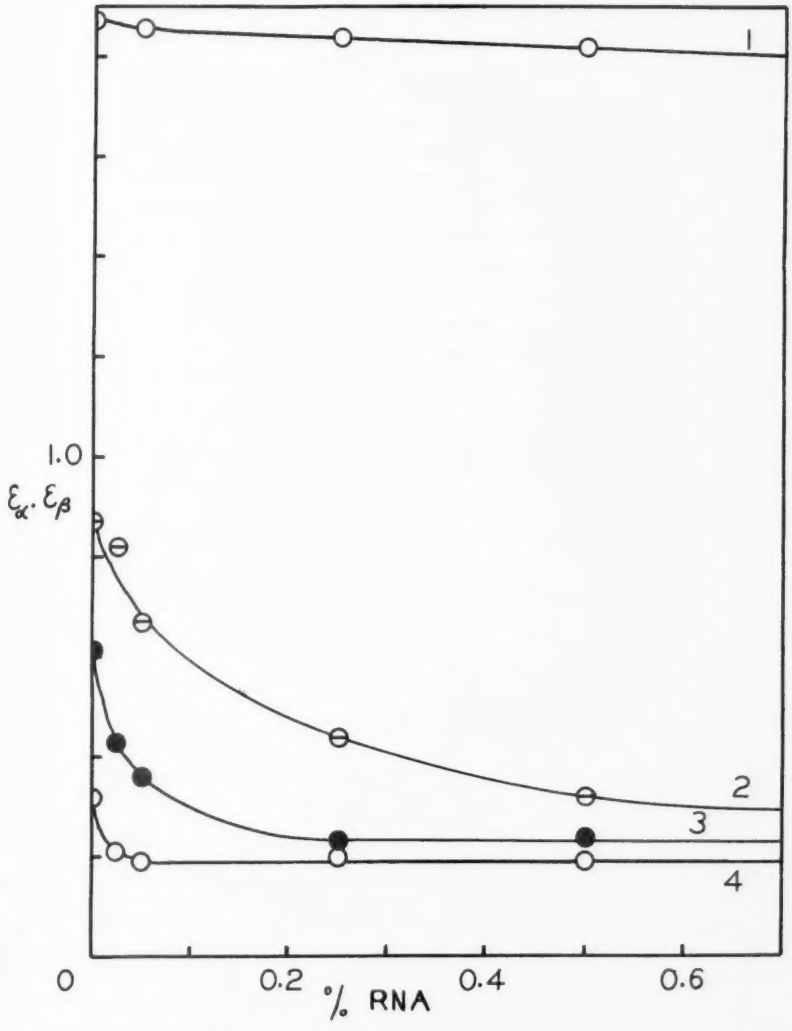

FIGURE 3. The relationship between nucleic acid concentration and the intensity of absorption bands of dyes. Ordinate = arithmetic product of the extinction coefficients of the alpha and beta bands of the spectrum. Abscissa = yeast ribose nucleic acid concentration. In all cases the dye concentration was 0.25×10^{-4} M, pH = 5. Curve 1 = rhodamine. Curve 2 = acridine red. Curve 3 = methyl pyronin. Curve 4 = ethyl pyronin.

of a basic dye is related, amongst other things, to the affinity with which the dye combines with depolymerized nucleic acid.

It might seem that rhodamine is an exception to this generalization, since it shows a mutagenic activity of a similar order of magnitude to that

of acridine red, yet the spectroscopic data indicate that it has little affinity for nucleic acid. Apart from the fact that the mutagenic activity of this dye is low and close to the border line of significance, the apparently anomalous activity could be related to the fact that the rhodamine molecule is highly diffusible in comparison with the other three xanthene dyes. From table 5 it may be seen that the two pyronins and acridine red show the usual inverse relationship between diffusibility and molecular weight, but in the case of rhodamine the diffusibility suddenly increases in spite of the large jump in molecular weight resulting from the addition of a further benzene ring in the molecule. The lipoid solubility of rhodamine is also very much greater than that of acridine red or of the two pyronins (unpublished observations). It therefore seems probable that rhodamine is able to cross cell boundaries with greater ease than can the other three dyes and thus the intracellular concentration of dye that can be attained

TABLE 5

RELATIVE DIFFUSIBILITIES OF DYES OF THE PYRONIN SERIES

Temperature = 25° C; pH 7.0; M/50 phosphate buffer; dye concentration of 0.5×10^{-3} M and 2% agar.

Dye	Molecular weight	Relative diffusibility
Ethyl pyronin	359	1.0
Methyl pyronin	303	1.7
Acridine red	274	2.6
Rhodamine	479	4.3

in the gonads may be of a sufficiently higher level to compensate in some measure for inherent mutagenic weakness. Certainly diffusibility and ease of passage across cell boundaries must be very important factors involved in the action of chemical mutagens of all kinds.

It may be noted that microinjection experiments on Amoeba have demonstrated that the nuclear membrane is freely permeable to many dyes (Monné, 1935; Clark, 1943). In Drosophila, by utilizing the fact that pyronin can be detected in very low concentrations by fluorescence microscopy, it can be shown that following injection into third instar larvae, the dye is present in detectable amounts within the nucleoli of salivary gland cells. Such injected larvae usually recover and may be used for genetic tests. Similarly, the dye can also be shown to accumulate in vivo or in vitro in rat tissues to give a histological picture resembling that obtained by pyronin staining of fixed material. Provisionally therefore it can be assumed that pyronin does react with non-polymerized nucleic acids within the living cell.

DISCUSSION

Although the evidence suggests that pyronin produces a genetic effect by combining directly with nucleic acid, it is not possible to draw any conclusions from the data as they stand as to the precise site of action. Assuming that Kurnick's finding, that pyronin reacts only with unpolymerized nucleic acid, is valid for the behavior of the dye in vivo, the mutagenic

activity might result from combination with desoxypentose nucleic acid at some stage in the synthesis of the latter when it is in a depolymerized state, or from combination with pentose nucleic acids, which do not seem to occur in the highly polymerized state. If the effect is due to combination with RNA, is the site of action the chromosome itself, which is known to contain a small amount of ribose nucleic acid, or is it the nucleolus, which seems to consist largely of RNA? Perhaps it is not impossible that the initial reaction takes place in the cytoplasm with some of the RNA-containing granules, the modified nucleic acid being incorporated into the nucleus at a succeeding division cycle. Jacobson and Webb (1951) find that RNA accumulates at the surface of the chromosomes at anaphase, only to be shed off again at late telophase. Since these authors did not observe any decrease in cytoplasmic basophilia during mitosis, it is perhaps rather unlikely that the cytoplasmic RNA would be incorporated to any great extent in the matrix of the chromosome. The recent observations of Walker and Yates (1952) on nucleic acid distribution in dividing cells also fail to support the view that there is a turnover from the cytoplasmic RNA to nuclear DNA during prophase. However the possibility that the primary action is cytoplasmic should be kept in mind.

As to the precise mode of action of pyronin, as distinct from the site of action, there appear to be at least two possibilities. The dye may combine with unpolymerized nucleic acid to form a complex which interferes with and brings about alterations to the spatial arrangement of nucleotides characteristic for a given locus when the nucleic acid becomes highly polymerized at a later stage in the mitotic cycle. Alternatively, if Haurowitz's (1950) suggestion of the role of nucleic acid is correct, perhaps union with pyronin prevents the nucleic acid from maintaining the "template protein" in an expanded state during protein synthesis. Since Mirsky and Ris (1951) find that the binding of basic dyes by nucleoprotein results in the displacement of protein from its combination with nucleic acid, this could perhaps result in an "error" in gene duplication that would be recorded in a genetic test as a lethal.

Recently, Briggs (1952) has shown that a number of basic dyes are able to inactivate frog sperm nuclei without interfering with the capacity of the sperm to fertilize eggs. Although Briggs finds that pyronin can incapacitate the sperm nucleus, so also can methylene blue and thionine, yet these latter two dyes show no signs of being mutagenic. It is probable, therefore, that the processes underlying the mutagenic action of pyronin in Drosophila and the inactivating effect of the dye in frog sperm are different.

That the biological activity of pyronin may be of special interest is suggested by an entirely different series of experiments recently made in this laboratory by Hoffman (1953), who has been studying the effects of the dye on the rate of reinnervation of partially denervated rat muscle. The idea behind these experiments was that when new sprouts are forming at the end of a nerve fibre there must be active synthesis of protein. Now since it is widely held that there is some close relation between protein synthesis and nucleic acids, it seemed worth while to ascertain whether

the administration of a dye such as pyronin would affect in any way the rate of reinnervation. Hoffman found that when the operated rats were given pyronin in their drinking water, there was an astonishing increase in the rate of reinnervation, the process proceeding about four times as rapidly as in the controls. The method did not allow of the detection of any marked difference between the activities of ethyl and methyl pyronin, but, as Hoffman has pointed out in a personal communication, the stimulating effect of both pyronins is so great that it would be difficult to distinguish histologically between an acceleration of the process and a marked acceleration. Other dyes tested by Hoffman (1953) were found to be inactive. It is therefore suggested that combination with unpolymerized nucleic acid is the common factor between the ability of pyronin to stimulate nerve sprouting in partially denervated rat muscle and to produce genetic effects in Drosophila.

SUMMARY

A number of basic dyes have been tested for ability to produce sex-linked, recessive lethals in *D. melanogaster*, the most active being pyronin B. It is suggested that the mutagenic activity of a dye is related, amongst other things, to its affinity for unpolymerized nucleic acid.

ACKNOWLEDGMENTS

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AGGLUTINATIONS OF THE ERYTHROCYTES OF VARIOUS FISHES BY HUMAN AND OTHER SERA

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The erythrocytes of certain species of fish are agglutinated by natural antibodies occurring in human sera (Cushing and Sprague, 1952). Analysis of the antigens involved in the agglutination of the erythrocytes of the white croaker or "kingfish," *Genyonemus lineatus* (Ayres), showed that it has an antigenic component on its cells similar to the human B antigen and a second antigenic component not related to the human B or A antigens. These components have been designated F-1 and F-2, respectively. The red cells of the shiner seaperch, *Cymatogaster aggregata* (Gibbons) were shown to have an antigen distinct from F-1 and F-2. In addition the shiner seaperch has an antigen that reacts with rabbit anti-sheep cell serum. These antigens have been designated F-3 and F-4, respectively. The antigens of both species appear to be species-specific in that they have not been found to vary individually.

The present paper outlines the results of a comparative study of the reactions, with human sera and with rabbit anti-sheep cell serum, of the erythrocytes of various fishes. The purpose of this study was to obtain information regarding the antigens of fishes that might be useful in studying their evolution, stocks, and migrations (Cushing, 1952a) and to learn something of the occurrence in fishes of the human A and B antigens.

MATERIAL AND METHODS

The methods of absorption and testing used in the present paper have already been described in detail (Cushing and Sprague, 1952). The methods of collection of fish erythrocytes were varied. With the exception of the rainbow trout, *Salmo gardineri* (Gibbons), all fish examined were of marine species. Most of the trout were obtained from the California State Hatchery at Fillmore, through the courtesy of Mr. C. W. Chansler; others were typed as they were caught, with the aid of Mr. Cambell Grant, in a stream near Santa Barbara. Anchovy were made available through the kindness of Mr. Walter Welton, owner of live bait receiving boxes at Hueneme, California. The other marine fish were obtained at the Scripps Institution of Oceanography at La Jolla, California, and near Santa Barbara. The majority of the fishes studied were taken in the vicinity of the Channel Islands off southern California during a week's trip aboard the research ship *Orca*. This trip immeasurably accelerated the present study and was only possible through

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the much-appreciated generosity of Mr. Joseph W. Sefton, Jr., owner of the *Orca*, who sponsored the Channel Island research.

As soon as they were caught, fish were bled from the heart by the use of suitable sized syringes. The blood was put into a sea-water Ringer's solution (3.5 per cent saline) containing heparin (50 mg. per 10 ml.), placed in screw cap vials, and transported to the laboratory for analysis. With exceptions noted below, the erythrocytes of most species, when collected in this way, last several hours, if kept cool and shaded.

Test antisera were prepared by suitable absorption and were kept frozen when not in use. Repeated freezing and thawing did not affect the titers of the sera. Agglutinations were observed by placing a drop of serum with a drop of two per cent washed cell suspension on a depression slide and reading the reactions by eye after five minutes of mixing. Readings were confirmed at fifteen minutes. They were recorded as 4+ for complete agglutination, 3+ for three-fourths of the cells agglutinated, 2+ for one-half of the cells agglutinated, + for weak but definite agglutination of some cells, and 0 for no agglutination. Conclusions were based on contrasts between strong (4+ and 3+) agglutination and no (0) agglutination. Controls were used throughout.

The cells of most species of fish studied were found to be stable for twelve or more hours. Erythrocytes of the jack-mackerel, *Trachurus symmetricus* (Ayres), however, hemolized the instant they were drawn. The cells of the ocean northern anchovy, *Engraulis mordax mordax* (Girard), were also unstable and not only had to be tested within an hour or so of collection, but hemolized rapidly in the test sera. Autoagglutination was encountered only in preparations from the thornback, *Platyrhinoidis triseriata* (Jordan and Gilbert), but it was possible to establish, by the use of suitable controls, that the cells of this species reacted negatively to the test sera.

THE VIVIPAROUS PERCHES

The family to which the shiner seaperch belongs was selected as a point of departure for comparative study. This family, Embiotocidae, often referred to as the "surfperches" or "viviparous perches," comprises several genera, of which Hyperprosopon, represented by the walleye surfperch, *Hyperprosopon argenteum* (Gibbons), was considered first. Reciprocal absorptions of rabbit anti-sheep red cell serum involving shiner seaperch, walleye surfperch and mammalian sheep cells established the presence of the F-4 component on walleye surfperch cells. Similarly, the F-3 component was also found and additional work shows that all the viviparous perches examined have these two antigens in common (table 1).

The viviparous perches were next compared (table 2) with the white croaker; jacksmelt, *Atherinopsis californiensis* (Girard), Pacific mackerel, *Pneumatophorus japonicus diego* (Ayres), and rainbow trout. Antigenically, jacksmelt appear to resemble the viviparous perches. Two new antigens must be introduced to explain the differences in the reactions of trout and

TABLE 1

ERYTHROCYTE ANTIGEN REACTIONS OF THE VIVIPAROUS PERCHES (EMBIOTOCIDAE)

These data indicate the uniformity of the reactions of the erythrocytes of species of viviparous perches to various anti-sera. That the absorbed sera were not over-absorbed is shown by the fact that they still gave 4+ reactions with the proper human cells, as well as the other reactions to be seen in succeeding tables. The reactions can be readily explained by assuming that the viviparous perches have in common the same two antigens.

Species providing erythrocytes	Non-absorbed sera			Absorbed sera					
	Anti-A	Anti-B	Anti-*Cymatogaster*	Anti-A with A cells	Anti-B with B cells	Anti-AB† with *Cymatogaster* cells	Anti-A with *Amphistichus* cells	Anti-B with *Amphistichus* cells	Rabbit anti-sheep cell serum
Walleye surfperch *Hyperprosopon argenteum*	4+	4+	4+	4+	4+	0	0	0	4+
Silver surfperch *Tocichthys ellipticus*	4+	3+	4+	4+	4+	0	0	0	4+
Barred surfperch *Amphistichus argenteus*	4+	3+	4+	3+	3+	0	0	0	4+
Shiner seaperch *Cymatogaster aggregata*	4+	4+	4+	4+	4+	0	0	0	4+
Black perch *Embiotoca sp.*	4+	4+	4+	....	....	0	0	0	4+
Pileperch *Damalichthys vacca*	4+	4+	4+	....	....	0	0	0	4+
Leastperch *Micrometrus minimus*	4+	4+	4+	....	....	0	0	0	4+
White seaperch *Phanerodon furcatus*	3+	+*	3+	2+	2+	0	0	0	4+

*Possible error.
†mixture of equal volumes of anti-A and anti-B.
.... no test.

TABLE 2

ANTIGEN REACTIONS OF VARIOUS FISHES

These reactions show the relationship of the antigens of several fish with absorbed antisera. They can readily be explained by assuming that the white croaker has antigens F-1 and F-2, the shiner seaperch, barred perch and jacksmelt have antigens F-3 and F-4, the Pacific mackerel has antigens F-4, F-5 and an "A" antigen and the rainbow trout antigen F-6. All fish are positive with unabsorbed anti-A and anti-B sera.

Species	A absorbed anti-A	B absorbed anti-B	Anti-Sheep* cell	Shiner seaperch absorbed anti-AB	Barred surfperch absorbed		Jacksmelt absorbed		Pacific mackerel absorbed		Rainbow trout absorbed		Rainbow trout and Pacific mackerel absorbed	
					anti-A	anti-B	anti-A	anti-B	anti-A	anti-B	anti-A	anti-B	anti-A	anti-B
Ocean Northern anchovy *Engraulis mordax mordax*	4+	4+	0	4+	....	....	....	....	4+	4+	....	....	....	....
Rainbow trout *Salmo gardineri*	4+	4+	0	3+	....	....	....	....	4+	4+	0	0	0	0
Jacksmelt *Atherinopsis californiensis*	4+	4+	4+	0	0	0	0	0	3+	3+	4+	3+	4+	3+
Kelp bass *Paralabrax clathratus*	2+	+	0	+	2+	+	3+	3+	+	0†	0	0	....	....
Pacific mackerel *Pneumatophorus japonicus diego*	4+	4+	4+	4+	4+	4+	4+	4+	0	0	4+	4+	0	0
White croaker *Genyonemus lineatus*	2+	+	0	+	2+	2+	2+	3+	2+	....	2+	3+	2+	4+†
Barred surfperch *Amphistichus argenteus*	4+	4+	4+	0	0	0	0	0	3+	3+	2+	3+	....	....
Shiner seaperch *Cymatogaster aggregata*	4+	4+	4+	0	0	0	0	0	3+	3+	4+	3+	3+	3+
Opaleye *Girella nigricans*	3+	3+	3+	+	4+	0	4+	0	0	0	4+	0	0	0
Halfmoon *Medialuna californiensis*	3+	+	4+	0†	4+	+	4+	+	+	0	4+	+	0	0
Redgarnet cabezon *Scorpaenichthys sp.*	3+	3+	3+	3+	....	....	....	....	3+	3+	4+	4+	....	....

* Not absorbed
† Possible error
.... No test

mackerel cells. Each of these species must be postulated to have at least one antigen specific to itself and the mackerel to have, in addition, an antigen resembling the human A substance. The consistency of the experiments, utilizing several tests on three or more fish of each species, justify the recognition of two more species-specific antigens, designated by number as F-5, on mackerel cells, and F-6, on rainbow trout cells. The mackerel A antigen is not sufficiently well characterized as yet to receive a numerical designation (see below).

THE ANTIGENS OF OTHER FISHES

As only one of each kind of fish was used in the supplementary determinations, excepting for the anchovy, no very definite conclusions were reached. However, the results (table 2) indicate that the opaleye, *Girella nigricans* (Ayres), and the halfmoon, *Medialuna californiensis* (Steindachner), have an "A" substance like that of humans, and that Pacific mackerel cells specifically absorb the antibodies in anti-A serum that are capable of reacting with this substance. The kelp bass, *Paralabrax clathratus* (Girard), appears to be like the trout in its antigenic constitution. Other fishes that reacted positively with non-absorbed human typing sera or with rabbit anti-sheep cell serum are listed below. Positive reactions are indicated by these letters followed by a "+" sign; anti-A = A; anti-B = B and anti-sheep cell serum = S. A negative response is shown by an "0" after the letter. Of these fish the California halibut, *Paralichthys californicus* (Ayres), is the only one that appears at first glance to have a human-like antigen (B). Reactions of different species range from weak (+) to quite strong (3+).

Species	Reaction
Yellowfin tuna, *Neothunnus macropterus* (Temminck and Schlegel)	A+, B+
Oceanic skipjack, *Katsuwonus pelamis* (Linnaeus)	A+, B+
Ocean-whitefish, *Caulolatilus princeps anomalus* (Cooper)	A+, B+, SO
Kelp rockfish, *Sebastodes atrovirens* (Jordan and Gilbert)	A+, BO, SO
California halibut, *Paralichthys californicus* (Ayres)	B+, AO, SO
Northern midshipman, *Porichthys notatus* (Girard)	A+, BO, SO
Buffalo sculpin, *Enophrys taurina* (Gilbert)	AO, BO, S+

Additional antigens on fish cells were indicated by the use of Jack bean extract, which strongly agglutinated the cells of California halibut and of the walleye surfperch, and weakly agglutinated the cells of the shovelnose guitarfish *Rhinobatos productus* (Ayres). Rabbit anti-serum against erythrocytes of the Pacific sardine, *Sardinops caerulae* (Girard), failed to agglutinate the cells of the California halibut and of the guitarfish, strongly agglutinated the cells of the halfmoon, and weakly agglutinated cells of members of the Embiotocidae.

NEGATIVE FISH

Cells of several species of fish failed to agglutinate in the presence of human anti-A, human anti-B, and rabbit anti-sheep cell serum. These are

listed below. The number following the name of each species shows the number of individuals that were tested.

Blue shark, *Prionace glauca* (Linnaeus) (2)
Soupfin, *Galeorhinus zyopterus* (Jordan and Gilbert) (2)
Shovelnose guitarfish, *Rhinobatos productus* (Ayres) (1)
Thornback, *Platyrhinoidis triseriata* (Jordan and Gilbert) (1)
California flyingfish, *Cypselurus californicus* (Cooper) (1)
Pacific sand dab, *Citharichthys sordidus* (Girard) (4)
Curlfin turbot, *Pleuronichthys decurrens* Jordan and Gilbert (1)
Blacksmith, *Chromis punctipinnis* (Cooper) (1)
Sheephead, *Pimelometopon pulchrum* (Ayres) (3)
Señorita, *Oxyjulis californica* (Gunther) (3)
Scorpionfish, *Scorpaena guttata* Girard (1)
Black rockfish, *Sebastodes melanops* (Girard) (2)
Roughback Sculpin, *Chintonotus pugetensis* (Steindachner) (1)
Pipefish, *Syngnathus* sp. (1)
Mudsucker, *Gillichthys mirabilis* Cooper (4)
Klipfish, *Gibbonsia* sp. (1)
Blenny, *Hypsoblennius* sp. (1)

THE RELATION OF FISH "A" ANTIGEN TO HUMAN "A" ANTIGEN

Several observations indicate that an antigen with a specificity resembling that of the human *A* antigen probably occurs in the erythrocytes of the mackerel. Human anti-*A* agglutinates mackerel cells faster than does human anti-B. Relatives of the mackerel, the yellowfin tuna, *Neothunnus macropterus* (Temminck and Schlegel), and the skipjack, *Katsuwonus pelamis* (Linnaeus), have an "A" antigen in their cells (Cushing, Sprague and Drake, unpublished data). Mackerel absorption of human anti-*A* removes the ability of this serum to agglutinate the erythrocytes of the opaleye and the halfmoon (these fish also appear to have "A" antigen on their cells, as shown by the reactions listed in table 2).

Whether or not mackerel "A" substance is responsible for the reactions of the cells of this fish with rabbit anti-sheep cell serum remains an open question. The problem will be studied further in connection with a detailed investigation of the cellular antigens of fish of the series Scombriformes (mackerel-like fishes). The nature of the absorption of human anti-*A* serum with cells of various species of this series will also be studied. The complex relationships of these antigens, already apparent, recalls the considerable heterogeneity that is known to exist within the human A, anti-*A* series of reactions, and also brings to mind the fact that certain rabbit anti-sheep cell sera agglutinate type *A* human cells (Wiener, 1943).

NATURAL HEMOLYSINS AND AGGLUTININS IN FISH SERA

Several incidental observations (table 3) on the agglutinin content of fish sera may have some taxonomic usefulness and bear on the demonstration of similar antibodies in tuna sera (Cushing, 1952a and 1952b).

There are marked differences in the agglutinin and hemolytic content of the sera of several species of fish. Grubb (1949) discusses agglutinins in eel serum for human cells that vary individually within this species and

TABLE 3

REACTIONS OF FRESH FISH SERA WITH VARIOUS MAMMALIAN ERYTHROCYTES INDICATING DIFFERENCES IN THEIR AGGLUTININ AND HEMOLYSIN CONTENT

All sera diluted 1 in 4 or less. Agglutination to any degree is indicated by +, hemolysis by H. Reactions are based on tests of one fish only, excepting where the word "pool" follows the species name. In these cases the sera of three to six individuals were combined.

Species	Erythrocytes					
	Human type:			Sheep	Guinea pig	Rabbit
	A	B	O			
Rainbow trout (pool) *Salmo gardineri*	O	O	O	O	....	....
Jacksmelt *Atherinopsis californiensis*	O	O	O	O	....	....
Pacific mackerel *Pneumatophorus japonicus diego*	O	O	O	O	....	....
Blacksmith *Chromis punctipinnis*	1. O	O	O	O	O	H
	2. O	O	O	O	O	O
	3. H	O	O	+	O	H
	4. O	O	O	O	O	H
Sheephead (pool) *Pimelometopon pulchrum*	H	H	H	O	....	....
Señorita (pool) *Oxyjulis californica*	O	O	O	O	O	H
Opaleye *Girella nigricans*	H	+	+	O	H	H
Halfmoon *Medialuna californiensis*	O	O	O	O	O	O
Black rockfish *Sebastodes melanops*	O	O	O	O	....	....

which may be of value in distinguishing serological types in the European eel, *Anguilla anguilla* (Linnaeus).

Hemolysis could be prevented by heat inactivating the complement concerned.

DISCUSSION AND SUMMARY

The erythrocytes of a variety of fishes have been found to react positively with normal antibodies in human sera. These reactions are due to several distinctive antigens, including the human A and B antigens and antigens found on sheep cells. The antigens are widely distributed throughout the class Pisces and appear to have been sorted into various combinations by the vagaries of evolution. Different combinations characterize groups of related species. The study suggests that either the human cellular antigens are evolutionarily very ancient and have survived with relatively little change throughout long periods of time, or that they readily

TABLE 4

A SUMMARY OF ANTIGENS THAT HAVE BEEN IDENTIFIED BY THE AUTHORS

The presence of an antigen is indicated by a + sign, absence by a 0 sign. An asterisk denotes that the determination needs to be confirmed by further study. The "A" antigen will not be assigned a number until its relations to the F-4 antigen are better understood.

Species of occurrence	F-1 ("B")	F-2	F-3	F-4 ("Forssman")	F-5	F-6	"A"
Rainbow trout *Salmo gardineri*	0	0	0	0	0	+	0
California halibut *Paralichthys californicus*	+*	....	....	0	....	....	0
Kelp bass *Paralabrax clathratus*	0*	0*	0*	0*	0*	+*	0*
Jacksmelt *Atherinopsis californiensis*	0	0	+	+	0	0	0
Pacific mackerel *Pneumatophorus japonicus diego*	0	0	0	+*	+	0	+
Oceanic skipjack *Katsuwonus pelamis*	....	....	....	....	....	....	+
Yellowfin tuna *Neothunnus macropterus*	....	....	....	....	....	....	+
White croaker *Genyonemus lineatus*	+	+	0	0	0	0	0
Shiner seaperch, etc. *Embiotocidae*	0	0	+	+	0	0	0
Opaleye *Girella nigricans*	0*	....	....	+*	....	....	+*
Halfmoon *Medialuna californiensis*	0*	....	....	+*	....	....	+*

*See legend for explanation of * in this table.

appear during the course of evolution in species that have either lost or have never had them.

The several antigens that have been characterized in fishes to date are listed in table 4, together with the species in which they occur.

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FLUCTUATIONS IN DROSOPHILA POPULATIONS IN A TROPICAL AREA

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Seasonal fluctuations in Drosophila populations have been studied by Dobzhansky and Epling (1944) near or on Mt. San Jacinto in California, by Patterson (1943) near Austin, Texas, and by Pipkin (1952) in the Lebanon Mountains and environs. The pronounced influence of climatic changes in regions possessing marked seasons has been reported by these investigators. Month to month fluctuations in Drosophila populations of tropical Brazil have been described by Dobzhansky and Pavan (1950). These authors suggest that "fruiting seasons, which occur even in areas of uniform climate, are apparently responsible for changes in the available food supply, hence in the numbers of flies supported by a given region." For a period extending over approximately fourteen months on Moen Island, Truk, Eastern Caroline Islands, the author had the opportunity of observing monthly fluctuations in Drosophila populations based upon almost daily collections. Some factors influencing population size in this tropical area were established.

Tiny volcanic Moen Island lies within the Truk lagoon at a latitude of 7° 27' N and a longitude of 151° 50' E. The collecting station was located at an elevation of about 10 meters on a hillside covered with high grasses, creepers, papaya *(Carica papaya)*, and hau *(Hibiscus tiliaceus)* trees. One large breadfruit tree *(Artocarpus altilis)* stood approximately 3 meters from the traps, no other breadfruit tree being within 50 meters of the collecting station. A dozen papaya trees, fruiting continuously, and several pineapple plants, none fruiting during the period of collection, were within 15 meters of the traps. Four one-gallon trap cans were placed inside a wooden box rain shelter adjacent to a large basalt boulder which served as a wind shield. The trap cans were baited with bananas, limes, papayas, and pineapples which are grown throughout the year on the island and also with mangoes and breadfruit which mature twice a year. Flies were netted between 9 and 10 o'clock each day. Collections were carried on from August 27, 1951, to June 4, 1952, and from August 11, 1952, to October 10, 1952.

The only marked change disturbing the tropical wet climate of Moen Island was a windy season from January through April. Mean monthly wind speeds in miles per hour beginning September, 1951, and extending through September, 1952, were as follows: 3.34, 4.32, 4.26, 5.64, 9.55, 10.24, 7.79, 7.04, 5.82, 5.47, 3.22, 3.20, and 4.62. For the period from September 1, 1951, through September 30, 1952, the monthly precipitation varied from 5.0 inches in February, 1952, to 18.3 inches in October, 1951. The total

rainfall for the twelve months beginning with September 1, 1951, was 125.9 inches. The rainfall was not markedly lowered during the January-April windy season. The monthly precipitation in inches from September, 1951, through September, 1952, was as follows: 8.45, 18.30, 7.83, 8.94, 10.13, 5.03, 7.38, 5.60, 11.32, 16.50, 11.90, 14.51, 11.98. Temperature and relative humidity percentages varied only slightly on Moen Island throughout the year. The range of mean monthly temperatures was from 79.8° F in August, 1952, to 81.5° F in December, 1951, for the thirteen months beginning September, 1951. Daily thermal variations were usually from 10–12° F. The mean monthly relative humidity percentages fell as low as from 75.4 to 78.3 during the windy season and ranged from 80.1 to 85.6 throughout the remainder of the thirteen months period beginning September, 1951. The meteorological measurements were made at the United States Department of Commerce Weather Bureau Station located at an elevation of 8 feet about one half mile from the Drosophila collecting station.

COLLECTION DATA FOR MOEN ISLAND

Only four Drosophila species were found in the traps on Moen Island: *D. ananassae, D. hypocausta, D. anuda,* and *D. melanogaster.* These species were also found on the Islands of Tol, Dublon, and Uman within the Truk atoll. No other species was found on these islands, although a search was made in fallen breadfruit, papayas, saur-sop (*annona muricata*), in fungi, and decaying wood.

Table 1 gives the collection data for Moen Island. The first column tells the number of collections per month. In rows corresponding to the respective months appear the average number of flies of each species collected per month. This figure is calculated, following Patterson (1943), by dividing the total number of flies caught in a month by the number of collections made in that month. The last column of table 1 indicates the presence or absence of fallen breadfruit from the big tree adjacent to the traps. This tree was bearing mature fruit during the whole of September, 1951, and continued to bear fruit until November 3, 1951, when the natives to whom the tree belonged cut and removed all fruit from the tree. In the spring of 1952, the first mature fruit fell on April 12, no more breadfruit falling until April 28. After this time, fruit was on the ground until May 20 when the crop was harvested as before. Other breadfruit trees on the island were in fruit at about the same time as the tree near the traps, but the exact time at which fruit became mature varied considerably. *D. ananassae, D. hypocausta,* and *D. anuda* were seen to feed upon the fallen breadfruit, and their larvae and pupae were found within it. At the height of the breadfruit season in September, 1951, many young flies of these three species were observed in the traps. Thus it is presumed that the fallen breadfruit served not only as food but as breeding grounds for *D. ananassae, D. hypocausta,* and *D. anuda.* The population of *D. melanogaster* was too small to determine if this species fed upon fallen breadfruit.

TABLE 1

COLLECTION DATA OF DROSOPHILA SPECIES ON KOEN ISLAND.

Year, month	Number collections	*ananassae*	*hypocausta*	*anuda*	*melanogaster*	Breadfruit on ground
1951						
Sept.	25	159.92	91.60	9.12	3.92	Yes
Oct.	19	64.95	80.42	19.68	0.63	Yes
Nov.	21	62.38	29.57	17.24	0.29	Harvested Nov. 3
Dec.	21	26.90	4.95	30.00	0.86	No
1952						
Jan.	26	2.00	0.11	1.96	1.88	No
Feb.	18	8.89	0.11	2.33	5.67	No
March	22	35.50	0.09	2.50	10.05	No
April	24	12.75	0.00	0.88	3.96	Many, beginning April 28
May	27	161.63	0.37	5.19	3.37	Yes, until harvested May 20
June 1–4	4	8.00	0.00	10.25	3.00	No
July	No collections					No
Aug.	19	21.05	11.79	5.57	0.05	No
Sept.	21	9.10	2.57	19.67	0.05	No
Oct. 1–10	8	2.63	2.50	10.50	0.00	No

Obvious competitors with Drosophilae for the fallen breadfruit were large numbers of the introduced giant African snail, *Achatina fulica*. These snails were seen devouring the fallen breadfruit amid swarms of Drosophilae. The voracity of the snails made necessary a daily renewal of the bait in the fly traps. The Micronesian Starling, *Aplonis opacus*, was likewise a competitor of Drosophilae in keeping the ripe papayas cleaned off the nearby trees. Few ripe papayas were ever found on the ground. The chief predatory enemy of the Drosophilae which visited the traps were small ants which made nests under the rain shelter containing the trap cans.

The monthly percentages of the three dominant Drosophila species, i.e., *D. ananassae, D. hypocausta,* and *D. anuda*, for the twelve months from September, 1951, to September, 1952, are presented in histogram form in figure 1. The period during June and July, 1952, during which the author was absent from the island, is represented by dotted lines on the graph.

According to table 1 and figure 1, *D. ananassae* reached a peak of population twice during the period studied, each peak coinciding with the time of mature breadfruit, i.e., in September and October, 1951, and in May, 1952. The number of *D. ananassae* per collection in November, 1951, was almost as high as in the preceding month although the breadfruit crop had

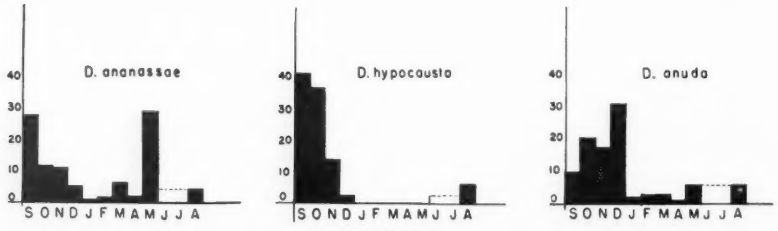

FIGURE 1

been harvested on November 3. A decline occurred from 62.4 flies per collection in November to 26.9 flies per collection in December, 1951. The numbers of this species entering the traps in January, 1952, decreased sharply to 2.0 per collection. The species expanded above minimal numbers, (i.e., 5 flies per collection) in February, 1952. Numbers of *D. ananassae* rose to 35.5 flies per collection in March, 1952, dipped to 12.8 flies per collection in April, 1952. The species underwent a peak of population in May, 1952, with 161.6 flies per collection owing to the stimulus of the ripening of the breadfruit on the big tree near the traps. The autumn fruiting of this tree was delayed in 1952, no mature fruit being on the ground by October 10, the date that collections were ended. Probably for this reason no expansion of the *D. ananassae* population was found from September, 1952, to October 10, 1952, in contrast with the presence of a large population of this species during the corresponding period of the previous year.

D. hypocausta underwent a sharp population expansion in September and October, 1951, corresponding to the maturing of breadfruit near the traps. Its population declined rapidly in November, reaching minimal numbers in December, 1951. Figure 1 shows that the species was extremely rare from January, 1952, to June, 1952. No population expansion paralleled the May breadfruit crop. A complete absence of *D. hypocausta* from the traps occurred from March 19 to May 8, 1952. The species occurred with a frequency of 11.8 individuals per collection from August 11 to August 31, 1952. The numbers were reduced to 2.57 flies per collection the following month and 2.50 flies per collection for the first ten days of October, 1952.

The population of *D. anuda* did not undergo such sharp expansions as *D. ananassae*. According to figure 1 and table 1, *D. anuda* was in a state of moderate expansion from September 1, 1951, through December, 1951. During the windy season from January through April, 1952, the population of this species dwindled to minimal numbers. In May, *D. anuda* averaged 5.19 flies per collection or barely above the level of minimal numbers. An increase in September, 1952, brought the average number of flies of this species up to 19.67.

D. melanogaster was never an abundant species on Moen Island, but it was found during every month of the period of collection. A peak of population was at no time reached. The species rose above minimal numbers in February and March, 1952, with 5.67 and 10.05 individuals per collection, respectively.

DISCUSSION AND CONCLUSIONS

A comparable influence of temperature, humidity, and rainfall changes upon population fluctuations of Drosophila as found in the Lebanon (Pipkin, 1952) did not exist on Moen Island. With a constant wet tropical climate, large variations in population size of *D. hypocausta* and *D. ananassae* at the collecting station on Moen Island appear to be owing almost wholly to the presence or absence of fallen ripe breadfruit from the tree near the traps. The autumnal (1951) and spring (1952) expansions of *D. ananassae* and the autumnal (1951) expansion of *D. hypocausta* were dependent upon the two fruiting seasons of the large breadfruit tree. The failure of both species to expand during the period from September 1 through October 11, 1952, was no doubt dependent upon the delay in maturation of the fruit on the large breadfruit tree near the traps at this time. These facts confirm the suggestion of Dobzhansky and Pavan (1950) that fruiting seasons in tropical areas are responsible for population fluctuations of certain Drosophilae. An absence of an expansion of the population of *D. hypocausta* in May, 1951, may be attributed both to its long 26 day life cycle and also to the fact that its population had declined drastically during the months prior to the brief spring fruiting season of the breadfruit tree. In contrast with *D. hypocausta*, *D. ananassae* was present in more than minimal numbers (more than 5 individuals per collection) from February through April preceding the May breadfruit season. With a 16 day life cycle, *D. ananassae* was thus able to take advantage of the increased food supply from April 28 until May 20, 1952. Upon the abrupt exhaustion of the breadfruit on May 20, owing to the harvest of all fruit on the tree by the natives and the consumption of fallen fruit within two days by the African snails, the population of *D. ananassae* declined catastrophically.

D. anuda was less influenced in its population size by the fruiting season of the large breadfruit tree than the preceding two species. Although present in well over minimal numbers in September and October, 1951, the time of the autumn fruiting of the big tree, the population of D. anuda continued to increase in December, 1951, long after the harvesting of the breadfruit by the natives on November 3. Taking into consideration the 18 day life cycle of this species, it seems nevertheless probable that the December rise in population numbers must have been dependent upon a different source of food supply than from the breadfruit tree near the traps. The population size of D. *anuda* suffered a sharp decline throughout the windy season from January through April, 1952, although *D. ananassae* both waxed and waned during different months of the windy season. It is possible that a drying effect of the wind influenced adversely the food supply of *D. anuda* which is known to include besides ripe fruits also the "bleeding sap" of trees. The breadfruit tree is itself a "bleeding tree."

The absence of a population expansion of *D. melanogaster* even during the breadfruit season may perhaps be attributed to the unfavorably high mean monthly temperatures of from 79.8° to 81.5° F existing the year round on Moen Island.

In conclusion it may be said that cyclic population expansions of some Drosophila species are to be expected in a wet tropical area where temperature and humidity are practically constant, depending upon the fruiting seasons of certain trees. Year to year variations in these cyclic expansions may be attributed to variations in the fruiting time of the trees, the fruit of which serves as Drosophila food supply and breeding grounds. Small, probably non-cyclic, month to month fluctuations of Drosophila populations, dependent upon irregular, local variations in food supply, may occur in the interim between fruiting seasons of major food supply trees.

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SUMMARY

Fluctuations in the populations of *D. ananassae*, *D. hypocausta*, *D. anuda*, and *D. melanogaster* were studied on Moen Island, Truk, Eastern Caroline Islands. Almost daily collections were made from fruit-baited traps for a period extending over approximately thirteen months. In this wet tropical area, with very little variation of temperature and humidity, the major population expansions of *D. ananassae* coincided with the autumn and spring fruiting of a large breadfruit tree near the fly traps. The population of *D. hypocausta* underwent a large expansion during the longer period of autumnal fruiting of the breadfruit tree but failed to expand during the spring fruiting. The population of this species had dwindled to very low numbers following the autumnal expansion. The highest population expansion of *D. anuda* occurred more than a generation's time subsequent to the autumn breadfruit harvest. The population of this species was probably dependent upon a source of food other than the breadfruit. *D. melanogaster* did not undergo any expansions during the period of collection. Small month to month fluctuations were observed in the population of *D. ananassae* during the period between the autumnal and spring fruiting of the big breadfruit tree near the fly collecting station. Cyclic large population expansions of a species in a uniformly tropical wet climate are believed to be dependent upon the fruiting of certain types of fruit trees. Small irregular month to month fluctuations of a species in such an area are probably non-cyclic but depend upon variations in the local food supply during the time between the fruiting of major food supply trees.

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A FERTILE INTERSPECIFIC HYBRID IN TRIFOLIUM
(4n *T. repens* L. × 4n *T. nigrescens* Viv.)[1]

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Attempts to produce interspecific hybrids in the genus Trifolium have been largely unsuccessful (Wexelsen, 1928; Guravich, 1949; Trimble, 1951; Keim, 1952). Trimble (1951) obtained one sterile hybrid plant from the cross of *Trifolium repens* (2n = 32) with *T. nigrescens* (2n = 16). A similar 24-chromosome hybrid was obtained in the present investigation (Keim, 1952). Keim (1953) has obtained and cytologically verified hybrids of *T. ambiguum* and *T. hybridum* utilizing an embryo culture technique. Naturally occurring hybrids of Trifolium species have been reported (Ascherson and Graebner, 1906; Hegi, 1925), although the hybrids were not investigated or verified cytogenetically.

The present paper concerns two fertile hybrids obtained from reciprocal crosses of colchicine-doubled plants of *Trifolium repens* L. and *T. nigrescens* Viv. Cytogenetic and morphological evidence verifying their hybrid origin is presented, together with preliminary data suggesting gene homologies of the two species.

MATERIALS AND METHODS

Plants of *T. repens* were grown largely from commercial lots of white and Ladino white clover seed. Seed of *T. nigrescens* was obtained from the Division of Forage Crops and Diseases, Bureau of Plant Industry, Soils, and Agricultural Engineering, Beltsville, Md. Methods of colchicine-induction and identification of tetraploids of these species have been outlined by Brewbaker (1952b).

Interspecific hybridizations were performed following suction emasculation of the florets, utilizing self-incompatible plants as females. Interspecies crosses set nearly 60 per cent as many seeds as intra-species crosses, although most of the seeds obtained were not viable. The two hybrid plants described here were obtained from seeds germinated on an agar medium (Keim, 1952).

DESCRIPTION OF PARENTAL SPECIES

The genus Trifolium comprises 250–300 species, of which *T. nigrescens* and *T. repens* are closely related species of the Section Euamoria, Subgenus Trifoliastrum (Ascherson and Graebner, 1906). *T. nigrescens* is a diploid (2n = 16), while *T. repens* (2n = 32) is one of six polyploid species

[1] Paper no. 297, Department of Plant Breeding, Cornell Univ., Ithaca, N. Y. Some parts of this paper are based on data included in the Ph.D. theses of both authors (Keim, 1952; Brewbaker, 1952a).

described in the genus (cf. Senn, 1938). Meiotic behavior in *T. repens* is highly regular, with no tetravalent association (Atwood and Hill, 1940).

Most of the plants in each species are self-incompatible. Nicotiana-type oppositional alleles at a single locus govern self- and cross-incompatibilities in each species (Atwood, 1940; Brewbaker, 1952a and unpublished data).

T. repens is perennial and spreads by means of stolons, while *T. nigrescens* is a sub-erect to erect annual. *T. repens* is an extremely polymorphic, cosmopolitan species. *T. nigrescens* occurs naturally in southern Europe and Asia Minor, and has recently been introduced into cultivation in the southern United States. In general, the leaves, flowers, seed, and pollen of *T. repens* are larger than those of *T. nigrescens*. Plants of both species display a variety of white leaf markings which appear to be conditioned in each species by multiple alleles at a single locus, with absence of marking recessive in each (Brewbaker and Anderson, 1952; Brewbaker, unpublished data). Anthocyanin spotting of the calyx tubes, a character which is apparently universal in *T. repens*, was observed only rarely in *T. nigrescens*.

Colchicine-doubled plants of these two species are commonly more robust than the standard types. Pollen grain size provides the most useful distinction between undoubled and doubled plants (Brewbaker, 1952b). Chromosome counts were made of plants in each of the undoubled and doubled lines of the two species, verifying the somatic numbers of 32 (4x) and 64 (8x) for *T. repens*, and 16 (2x) and 32 (4x) for *T. nigrescens*.

DESCRIPTION OF THE HYBRIDS

The two hybrid plants to be described were the first to mature among several successfully grown from crosses of doubled and undoubled plants of *T. repens* and *T. nigrescens*. The germination of seeds and excised embryos on agar and the characteristics of seedlings obtained from these crosses are described by Keim (1953). The two hybrid plants WN2 and WN3 were not studied cytologically. However, somatic chromosome counts were made of some of their hybrid and inbred progeny. In most of these plants, 2n = 48 was observed.

A. *Hybrid WN2*. The female parent of WN2 was a colchicine-doubled self-incompatible plant of *T. nigrescens* which had no leaf marking. The male parent was a colchicine-doubled plant of *T. repens* which was self-compatible and was homozygous for a dominant leaf marking allele.

The hybrid WN2 was self-compatible. It was prostrate and rapidly spreading, with slender stems and long internodes. Rooting at the nodes, a characteristic of the stoloniferous *T. repens* parent, was not observed in the hybrid. The leaves of WN2 exhibited the dominant leaf marking of the male parent. Among the 50 selfed progeny of WN2, two 48-chromosome plants had no leaf marking. Among the 28 testcross progeny of WN2 to doubled parental clones which lacked markings, one 56-chromosome and two 40-chromosome plants similarly lacked the markings. The rapid growth, profuse flowering, small leaves, and absence of anthocyanin calyx spots of WN2 suggested its affinities to the *T. nigrescens* parent.

The pollen of WN2 was ca. 90 per cent stainable. As male parent, WN2 was cross-fertile with WN3, doubled *T. repens*, and doubled *T. nigrescens*. It was cross-sterile as male to undoubled plants of the parental species. Crosses of WN2 as female to WN3 and to doubled *T. repens* as males averaged 11.7 and 11.0 plump seeds per ten flowers, respectively. Heads of WN2 which were tripped to effect self-pollination, using ten flowers per head, averaged 3.3 seeds per ten flowers. Entire heads self-pollinated averaged 22.3 seeds per head.

B. *Hybrid WN3*. The female parent of WN3 was a self-incompatible plant of doubled *T. repens* with full V leaf marking. The male parent was a colchicine-doubled self-incompatible plant of *T. nigrescens* which had a broken V marking with red tip.

The hybrid WN3 set no seed upon self-pollination. It showed the superimposed but separated leaf markings of both parents.

WN3 showed greater morphological affinity to the *T. nigrescens* parent than did WN2. It died after producing a single erect stem. Flowers were intermediate to those of the parents in size, and had no calyx spots.

WN3 produced ca. 75 per cent stainable pollen, and it was cross-fertile as male to WN2 and to plants of both parental doubled lines. Crosses of WN3 as female to WN2 averaged 12.5 seeds per ten flowers.

DISCUSSION

From their observation of the regularity of meiosis in 32-chromosome *Trifolium repens*, Atwood and Hill (1940) concluded that this species was probably an amphidiploid. Considering the results presented here, it is suggested that 16-chromosome *T. nigrescens* may be one of the ancestors of *T. repens*. The hybrids described are among the first if not the only fertile interspecific hybrids obtained in the genus. While *T. repens* has been crossed to at least twenty other Trifolium species, mature hybrids have been reported only from the cross with *T. nigrescens* (Wexelsen, 1928; Guravich, 1949; Trimble, 1951).

The preliminary data presented indicate probable gene homologies of multiple allelic loci conditioning intra-specific incompatibilities and leaf markings, respectively, in the two species genomes. The homology of the oppositional loci of the two species is suggested by the self-compatibility of hybrid WN2. This hybrid resulted from the cross of a self-incompatible plant of doubled *T. nigrescens* with a self-compatible plant of doubled *T. repens*. The self-compatibility of the *T. repens* parent resulted from the uninhibited growth in self-pollinations of pollen with a certain oppositional allelic combination ("competition" pollen; Atwood and Brewbaker, 1953). The self-compatibility of WN2 could be explained similarly by its production of the competition pollen class. The ability of WN2 to produce such pollen, containing both oppositional alleles of the *T. repens* parental genome, requires the assumption of random or between-species pairing of chromosomes of the two parental species. Preliminary data from advanced generation of hybrids WN2 and WN3 support this assumption.

Similarly, the homology of the leaf marking loci in the parental species is suggested by the segregation of unmarked plants in the progenies of WN2. WN2 had a white leaf marking, having obtained two dominant alleles from its marked *T. repens* parent, and two recessive alleles from the unmarked *T. nigrescens* parent. The homozygous recessive, unmarked hybrid and inbred plants of WN2 would not have been expected if the parental chromosomes preferentially paired within species. The data can be explained by assuming that the chromosomes which carry the V loci of the parental species paired essentially at random in species hybrid WN2.

Cytological and genetic investigations of I_1, F_1, and backcross families of these hybrids are in progress to elucidate the relationship of the parental species.

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SUMMARY

Two fertile hybrid plants were obtained from reciprocal crosses of colchicine-doubled plants of *T. repens* (2n = 64) and *T. nigrescens* (2n = 32). F_1 and I_1 progeny of these hybrids had 2n = 48. Cytogenetic evidence concerning the hybrids suggests that *T. nigrescens* may be one of the ancestors of amphiploid *T. repens*.

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SEGREGATION RATIOS OF MUTANT ALLELES FROM WILD POPULATIONS OF *Mus musculus*

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Several populations of wild house mice have been shown to contain animals heterozygous for recessive alleles (t^w) at locus *T* (Dunn and Morgan, 1953). The presence of the alleles is detected by mating animals captured in the wild to members of a laboratory stock having the short tail (Brachyury) mutation $T/+$, and recovering tailless lines T/t^w. Of five such alleles analyzed, four (t^{w1}, New York 1; t^{w3}, Connecticut; t^{w4}, Wisconsin; t^{w5}, New York 2) have proved to be lethal; one (t^{w2}, New York 1) is viable but ten homozygous males tested have been sterile. Each of these alleles segregates normally in female heterozygotes T/t^w or $+/t^w$, but male heterozygotes always produce an excess of t^w sperm. Ratios obtained by testing males T/t^w (tailless) by normal females are shown in table 1.

In these tests only about 5 per cent of the offspring are short-tailed, showing that they have received the *T* allele. Of the remaining 95 per cent, which are normal-tailed, all or nearly all have received the t^w allele. That this phenotypic ratio represents the genotypic ratio of $T/+$ to $t^w/+$ is shown by progeny tests of samples of offspring of this and of similar test crosses.

TABLE 1

RESULTS OF TEST-MATINGS OF TAILLESS MALES WITH DIFFERENT t^w ALLELES DERIVED FROM WILD MICE BY NORMAL-TAILED FEMALES (BAGG ALBINOS)

Males tested	Source	Progeny from +/+ females	
		Normal ($+/t^w$)	Brachy ($T/+$)
5 T/t^{w1}	New York 1	205	39
5 T/t^{w2}	New York 1	249	6
5 T/t^{w3}	Connecticut 1	352	3
4 T/t^{w4}	Wisconsin 3	115	2
5 T/t^{w5}	New York 2	207	14
Totals		1128	64

As shown in table 1, there are variations in the ratios obtained from the different samples of males. Usually males with the same t^w allele do not show consistent heterogeneity in respect to this ratio; while males T/t^{w1} and T/t^{w5} give higher frequencies of $T/+$ offspring (11.4 per cent) than do T/t^{w2}, T/t^{w3}, and T/t^{w4} males (1.5 per cent). There may be factors other than the nature of the specific t-allele involved which influence this ratio. These have not been studied. It is also likely that the segregation ratio of t^w depends in part upon whether it is segregating from the + or *T* allele. Groups of $+/t^w$ sons from the test crosses shown in table 1 are being tested

by crosses with $T/+$. It is already evident that the ratio advantage of t^w is less in the $+/t^w$ sons than in the T/t^w fathers. However there is in all cases a considerable preponderance of t^w sperm.

Such a segregation ratio advantage of a t^w allele should lead it to spread through any population in which it occurs in spite of the handicaps of lethality and sterility (Dunn, 1953; Prout, 1953). Assuming even moderate mutation frequencies of + to t^w we should thus expect to find t^w alleles in wild populations generally unless their spread is opposed by other circumstances.

TABLE 2

RESULTS OF TESTING CAPTURED WILD MICE BY CROSSING WITH BRACHY $T/+$

Source of sample	Animals tested	Progeny		
		Normal	Brachy	Tailless
Lawrence, Kansas	4 females			
(Prof. J. A. Weir)	3 males	60	48	
Norwich, Vermont	1 female			
(Dr. M.T.M. Rizki)	4 males	49	44	
Austin, Texas*	2 females	6	3	
(Prof. Frank Blair)				
New York, N. Y.	2 females	13	8	
(W. 247 St.)				
Sarasota, Florida	2 females	10	5	
(Mr. S. P. Holman)	6 males	50	45[a]	4
	1 male[b]	3	....	2
	F_1 male $+/t^{w6}$	2	....	5

[a]Many short Brachys intergrading with tailless
[b]Source of t^{w6}
*t^{w7} has been found in this stock.

Through the kindness of several colleagues who have sent us mice captured in the wild, we have been enabled to test samples of several populations additional to those reported earlier (Dunn and Morgan, 1953). The results are shown in table 2.

The results for the Vermont and Kansas samples are clearly negative, and there was no evidence in these populations of modifiers reducing the tail length of the Brachy phenotype.

The Florida population was less suitable for recovery of t^w alleles since, like two Wisconsin populations previously reported (Dunn and Morgan, 1953), it produced Brachy test offspring with very short tails, and these formed a continuous distribution with the few tailless offspring. One of these tailless exceptions has been tested and found to be $T/+$, that is, a phenotypic variant. However, one wild male gave only normal and *tailless* progeny, and one of these F_1 normals has been shown to be $+/t^{w6}$. Tailless progeny from the Florida population probably contain two genotypes, $T/+$ and T/t^{w6}, thus making it difficult to estimate the frequency of t^w alleles in this population. We are safe in saying that it contained at least one $+/t^w$ male out of seven tested. Judging from the frequency of Brachys obtained from the other six males tested, they are probably $+/+$.

The result of testing 25 mice captured in different parts of the United States is thus to show that t^w alleles are certainly not universal or even common in the wild populations; and where they are found, the heterozygotes $+/t^w$, in this as in previous tests, are in the minority as compared with +/+.

There is thus revealed a situation in which t^w alleles have been shown to enjoy a considerable segregation ratio advantage, and yet have not the frequency in wild populations to which this advantage should entitle them. It may be assumed therefore that other factors interfere with the spread of t^w alleles. These may be local ecological factors which discriminate against t^w heterozygotes, or genotypes favored locally for other effects which interact unfavorably with t^w alleles; or such heterozygotes generally may be at a disadvantage in competition with +/+, that is to say, t^w alleles may have dominant deleterious effects. The latter possibility is now being tested in a special experiment.

SUMMARY

Five mutant alleles found in different populations of wild mice have been shown to segregate from male heterozygotes in ratios greatly exceeding 50 per cent. This leads to the expectation that they should be common in wild populations. Tests of wild mice from five populations additional to those reported previously have revealed only two additional alleles in two populations. It is assumed that other factors oppose the spread of such alleles.

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LETTERS TO THE EDITORS

Correspondents alone are responsible for statements and opinions expressed. Letters are dated when received in the editorial office.

GENETIC EQUILIBRIUM WHEN MORE THAN ONE ECOLOGICAL NICHE IS AVAILABLE

In recent years the attention of experimental evolutionists has been increasingly directed toward polymorphism as furnishing desirable plasticity to a species. In particular, attention has been directed toward polymorphism with a known genetic basis. The best studied case is that of two alleles showing balanced polymorphism: that is, the heterozygote has a higher adaptive value in a certain environment or range of environments than either homozygote. Such balanced polymorphism is the only way a pair of alleles can remain in equilibrium within a single environment (or ecological niche), if we ignore mutation pressure and migration from the outside. On the other hand, it would seem that the existence of several ecological niches, with one allele favored in one niche and the other allele favored in another, might increase the possibilities for attainment of equilibrium with both alleles present in substantial proportions. Recently the question arose of whether it was in fact possible to have equilibrium without the heterozygote being superior to both homozygotes in any single niche. It is shown below that under certain assumptions the answer is yes.

The model here proposed is as follows: Let there be alleles A and A′ with gene frequencies of q and $1 - q$ respectively, and let mating be at random over the whole population, so that the initial zygotic frequencies are q^2AA, $2q(1 - q)$AA′, and $(1 - q)^2$A′A′. After fertilization the zygotes settle down at random in large numbers into each of the niches, and are thereafter immobile. There is then differential mortality ending with a fixed number of individuals in each niche. After selection the relative frequencies of AA, AA′, and A′A′ will be $W_1q^2 : 2q(1 - q) : V_1(1 - q)^2$ in niche 1, $W_2q^2 : 2q(1 - q) : V_2(1 - q)^2$ in the second niche, etc., where W_i and V_i are the adaptive values of AA and A′A′ individuals relative to AA′ in the i-th niche. We need consider only intra-niche comparisons and not the absolute viabilities in the different niches. If we disregard drift and consider only the force of selection, the absolute number of survivors in the different niches is also irrelevant and we may work with the numbers c_i, where c_i is the proportion of the total survivors to be found in the i-th niche, and $\Sigma c_i = 1$. To complete the model, we suppose that at the time of reproduction the survivors leave the niches, and that mating is at random in the entire population. If we denote by q′ the frequency of A in this mating popu-

lation, then $q' = \Sigma c_i q_i$, where q_i is the frequency of A in the i-th niche after selection.

It can easily be seen that under this model Δq is the weighted mean of the Δq's for the individual niches, so that

$$1) \qquad \Delta q = q' - q = q(1-q)\Sigma c_i \frac{(1-V_i) + (W_i + V_i - 2)q}{V_i + 2(1-V_i)q + (W_i + V_i - 2)q^2}$$

The factor $q(1-q)$ gives a trivial equilibrium at $q = 0$ and $q = 1$. The function $h(q) = \Delta q/q(1-q)$ will have the same sign as Δq for $0 < q < 1$. Since $h(q)$ is continuous, if $h(0)$ is positive and $h(1)$ is negative, there will be at least one q between 0 and 1 for which $h(q) = 0$ and hence at least one point of stable equilibrium. Setting $q = 0$ we find $h(q) = \Sigma c_i(1 - V_i)/V_i$, which is positive if

$$2) \qquad 1 + \Sigma c_i \frac{1 - V_i}{V_i} \equiv \Sigma c_i \frac{1}{V_i} > 1.$$

Setting $q = 1$ we find $h(1) = \Sigma c_i(W_i - 1)/W_i$, which is negative if

$$3) \qquad 1 - \Sigma c_i \frac{W_i - 1}{W_i} \equiv \Sigma c_i \frac{1}{W_i} > 1.$$

Conditions 2 and 3 are equivalent to the conditions that the weighted harmonic means of the W_i and of the V_i (the reciprocals of expressions 2 and 3) be less than one. Since the harmonic mean is less than the arithmetic mean except when all the numbers being averaged are equal, there will, *a forteriori*, be a stable equilibrium if the weighted arithmetic means of the W_i and V_i are less than one. For a single niche, this reduces to W_i and V_i both less than one, which is known to be a necessary and sufficient condition for a stable equilibrium. For more than one niche, conditions 2 and 3 are sufficient but not necessary. For example, with two niches and $c_1 = c_2 = \frac{1}{2}$, $W_1 = 2$, $V_1 = 1.1$, $W_2 = 0.5$, $V_2 = 1.1$, if initially $0 < q < 0.6$, equilibrium will be reached with $q = 0.35$, while if $q > 0.6$, A′ will be eliminated; in other words 0.35 is a point of stable equilibrium and 0.6 is a point of unstable equilibrium. In this example, the harmonic mean of V is $1.1 > 1$. On the other hand, with $c_1 = c_2 = \frac{1}{2}$, $W_1 = 2$, $V_1 = 1.2$, $W_2 = 0.5$, $V_2 = 1.2$ there is no equilibrium point between zero and one. An example fulfilling conditions 2 and 3 is $c_1 = c_2 = \frac{1}{2}$, $W_1 = \frac{3}{2}$, $V_1 = \frac{2}{3}$, $W_2 = \frac{2}{3}$, $V_2 = \frac{3}{2}$, giving a stable equilibrium at $q = \frac{1}{2}$. Note that in this example the weighted arithmetic means are greater than one, although the weighted harmonic means are less than one. For this last example the location of the equilibrium point at $\frac{1}{2}$ can be found by considerations of symmetry, but in general the actual value of the equilibrium point must be found by trial and error or some other approximate method.

The model here proposed is obviously not realistic; however, if it is modified by supposing that individuals move preferentially to niches they are better fitted for, or that there is a tendency for mating to occur within

a niche rather than at random over the whole population, conditions will be more favorable for equilibrium, so that in a sense we are considering the worst possible case. For another rather artificial model with variable population size, which will be discussed elsewhere, equilibrium is attained under similar conditions.

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PUBLICATIONS RECEIVED

THE AMERICAN NATURALIST is glad to acknowledge here the receipt of books on biological and natural history subjects which are likely to be of interest to our readers. No undertaking to publish reviews is implied in this acknowledgment. Books for notice may be sent to:

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Bent, Arthur Cleveland, 1953. Life histories of North American wood warblers. Smithsonian Institution United States National Museum Bulletin 203. 734 p. $4.50. U. S. Government Printing Office, Washington, D. C.

Bertrand, A., 1953. Succulent plants. 112 p., 10 color plates, 20 monochrome plates. $4.75. Philosophical Library, New York.

An account of the cultivation, propagation, enemies, and families of the succulent plants. One chapter each is devoted to the Agavaceae, Liliaceae, Asclepidaceae, Crassulaceae, Euphorbiaceae, and Arizoaceae. One oddity should be noted—a beautiful colored plate of *Stapelia Hirsuta* is pasted on the wrapper cover but does not occur in the book itself. Fifty-five different species are illustrated.

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Contains nine papers by as many authors on various phases of science today in the U.S.S.R., as first read at a symposium of the American Association for the Advancement of Science, December 27, 1951, and published in the Bulletin of the Atomic Scientists, Science, and Scientific Monthly.

Crocker, William, and Lela V. Barton, 1953. Physiology of seeds. 267 p., ill. $6.50. Chronica Botanica Co., Waltham, Massachusetts.

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III. Cornell's colors—carnelian and white. Studies in history No. 17. 7 p. $.30 paper. A. H. Wright, Ithaca, New York.